NUREG/CR-0684
UCRL-52481

SENSITIVITY OF MACROCYSTIS GAMETOPHYTES TO COPPER

Barbara M. Smith
Florence L. Harrison

Prepared for
U. S. Nuclear Regulatory Commission
by
Lawrence Livermore Laboratory

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Manuscript Submitted: August 4, 1978
Date Published:

Prepared for
Nuclear Regulatory Research, SAFER
U.S. Nuclear Regulatory Commission
Washington, D. C. 20555
Under Interagency Agreement DOE 40-550-75
NRC FIN No. A 0119
by
Lawrence Livermore Laboratory
Livermore, CA 94550

operated by University of California

for the U.S. Department of Energy

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ABSTRACT

Gametophytes of giant kelp, *Macrocystis pyrifera*, were exposed to copper in a dish-culture bioassay system. Through their development into gametophytes, newly released spores were exposed continuously to various concentrations of copper to determine its effect on vegetative growth. In addition, gametophytes of different ages were exposed continuously to copper to determine the sensitivity of several developmental stages to copper and the effect of copper on the production of gametes. A one-week pulsed-exposure experiment was performed to determine the recovery period for vegetative growth.

A counting procedure was developed, using a variation of the line-intercept method, to monitor the three-dimensional growth of the gametophytes. In experiments using copper spikes of less than 50 ppb, water samples were analyzed by differential pulse anodic stripping voltammetry to determine the content of labile copper.

Complete inhibition of growth occurred when gametophytes were exposed continuously to copper concentrations of 500 ppb or more. Vegetative growth was inhibited significantly by continuous exposure to 50 ppb copper; the production of eggs by female gametophytes was inhibited by spikes of 30 ppb copper, of which 1 ppb was ASV-labile. The growth of 1-, 2-, and 5-wk-old gametophytes was inhibited significantly by a 1-wk exposure to 100 ppb copper. The spiked cultures required 2 to 3 wk to recover from pulsed exposures to concentrations between 50 and 200 ppb copper.

INTRODUCTION

The total copper concentration in seawater is neither static nor uniform. Variations in the reported concentration of copper can result from several sources. The water mass that is sampled and the mixing conditions at the time of the sampling (Slowey and Hood, 1971), as well as the proximity of the site to particular land masses (Boyle and Edmond, 1975) can influence copper concentration. Site location near a region of upwelling (Knauer and Martin, 1973) also can affect the obtained measurement. The amount of organic material and whether it is particulate or colloidal can affect the amount of metal held and detected at the surface (Slowey and Hood, 1971; Chester and Stoner, 1974; Morris, 1974; Helz, Hugget, and Hill, 1975). How effectively a particular ion competes with other ions for a binding site on a ligand also may influence its speciation and, ultimately, its position in the water column (Seeley and Hart, 1974; Morris, 1974).

World-wide measurements of the total copper concentration in sea water—as distinguished from the concentration of soluble or ionic copper and particulate or chelated copper—range from less than 1 to 3 ppb copper (Spencer and Brewer, 1969; Boyle and Edmond, 1975). Measurements of copper taken along the California coast vary from 0.5 to 6.0 ppb, as determined by Dorband (1975), or from 1.0 to 1.7 ppb, as recorded by Knauer and Martin (1973). The background copper level for the influent

cooling waters at the Diablo Canyon power plant ranges from 1 to 3 ppb total copper (Martin, Stephenson, and Martin, 1974).

Copper and Algae

Naturally occurring copper is an important micronutrient for marine algae. A specific growth requirement for copper has been demonstrated both in *Chlorella vulgaris* and in *Oocystis marssonii* (Manahan and Smith, 1973), supporting the argument that the availability of metal micronutrients directly influences the cycles of phytoplankton growth (Neilsen and Wium-Anderson, 1970; Dunstan, 1975). Analysis of the metal content of brown algae indicates that, in polluted and unpolluted water, these plants accumulate metal ions both actively and passively (Yamamoto and Ishibashi, 1972; Bryan and Hummerstone, 1973). This accumulation provides algae with an important reserve of metallic components.

Copper and other metals that, in microquantities act as growth stimulants, depress growth in high concentrations (Nielsen and Wium-Anderson, 1970). The use of copper sulphate to depress algal blooms in eutrophic lakes is well documented (Hutchinson, 1957). In addition, elevated copper levels have been blamed for abalone deaths observed near the Diablo Canyon outfall (Martin, 1974).

Despite the foregoing, it is not adequately descriptive to define the copper toxicity as an interference with life processes. To date, considerable research has been devoted to measuring total copper concentration in seawater to evaluate how much metal the plants "see." However, it is not just the total concentration of copper, but also the chemical form of the metal (ionic or chelated) that influences copper toxicity. Khailov (1964) demonstrated that the medium in which several types of unicellular algae were grown had an increased ability to complex copper ions by hydrogen bonding to nonprotein-peptide fractions of yellow-stained compounds, thus decreasing the concentration of available copper. Humic substances were found to stimulate the growth of three *Gonyaulax* species; this was attributed partially to the chelation of metallic components in the test medium by these organic extracts (Prakash and Rashid, 1968). Experiments using *Ectocarpus siliculosus*, grown in media with and without the chelating agent Na_2EDTA, showed that growth supression occurs at lower copper concentrations when an inorganic medium is used than when the medium contains a chelator (Morris and Russel, 1973).

Copper Toxicity to Algae

Laboratory testing of copper toxicity has focused mainly on the effects observed in unicellular algae. Because much of what is known about the action of copper in an algal system comes from experiments on a single type of photosynthetic and physiological make-up (e.g., Chlorophyta, Bacillariophyta), conclusions drawn from these studies are not directly applicable to plants with different characteristics (e.g., Phaeophyta). However, the copper toxicity response in algae probably follows a similar mode of action in all plants.

In relatively low concentrations, ionic copper depresses photosynthesis. However, static cultures of algae often can exude enough fixed carbon to chelate the copper and thus effectively alter the ionic content of the medium (Neilsen and Wium-Anderson, 1970; Morris and Russel, 1973; Overnell, 1975). The toxic response of the organism to ionic copper is neither static nor uniform. Similar types of algae respond very differently to the same concentration of metal ions (Jensen and Rystad, 1976); also, the algae can exhibit different antagonistic or synergistic toxic responses to combinations of metal ions (Bryan, 1969; Sick and Windom, 1975). The toxic effect can vary even in a single type of algae with the compound to which the copper ion is complexed (Khobet'ev, *et al.*, 1975).

The effects of copper on the growth and photosynthesis of large groups of marine and estuarine microalgae and the possible use of these algae as indicator species have been the subject of considerable research (Berland, 1976; Erickson, 1970). Although certain genera or classes of algae generally are found to be more or less sensitive than others, there is a wide variation in sensitivity to copper among members of each taxonomic group. Some of this variation may result from the type of test medium selected, but much variability can be ascribed to the organisms themselves. Dinoflagellates, for example, generally appear to be more sensitive to copper than diatoms, although several diatom species are at least as sensitive as the dinoflagellates (Berland, 1976; Erickson, 1970).

Using two unicellular species, *Thallassiosira* and *Nannochloris*, Sunda and Giullard (1976)

calculated that the inhibitory concentration of ionic copper is between 10^{-9} and 10^{-11} *M*. Using *Chlorella*, Nielsen (1969) showed that the primary effect of an exposure to 50 ppb copper on the rate of photosynthesis is a decrease of the maximum rate at light saturation. This toxic effect can be reduced by the addition of sodium and potassium ions, as well as by the addition of citric acid, a weak chelating agent. Using *Dunaliella* and *Phaeodactylum*, Overnell (1975) demonstrated that increased adsorption of copper on the cell wall results in increased potassium loss from the cell; Overnell hypothesized that this loss of potassium ions resulted from a loss of cell membrane integrity.

Previous research on the copper toxicity in macroscopic brown algae has focused mainly on the effect of copper on growth (as measured by weight or size increase) and on the metal concentration in the plant tissue. Brown algae are known to concentrate metals in proportion to the concentration of the metals in the surrounding medium. Bryan (1969) examined this response in *Laminaria digitata*. Bryan found that a copper concentration of 50 ppb causes a reduction in the growth rate of zinc-exposed plants and that 200 ppb copper is lethal to these zinc-exposed specimens. Bryan also found that, at a concentration of 500 ppb, copper causes the rapid loss of accumulated zinc. One explanation of this phenomenon may be that copper binds to the alginate in the cell wall more readily than does zinc. A second explanation is that zinc solubilizes from the dead cell material that is released by the copper-killed cells.

Preston, *et al.* (1972) showed that *Fucus* contained from 1.7 to 28.4 ppm dry weight of copper; this represents a mean concentration factor of 4.5×10^3 times the ambient water concentration. By sampling and analyzing five areas around the British Isles, Preston, *et al.*, concluded that this alga would be a good indicator species. Using a sampling regime in which six sites were sampled eight times over one year, Morris and Bale (1975) found that the concentration of copper in *Fucus vesiculosus* in the Bristol Channel ranged from 3.82 to 12.0 ppm dry weight, representing concentration factors from 1.0 to 1.6×10^4 times the ambient water concentration, following the seaward-to-landward gradient of increasing copper concentration.

Seeliger and Edwards (1977) measured the copper content of the water and various algal species, including *Enteromorpha*, *Ulva,* and *Fucus*, and determined concentration factors ranging from 0.36 to 1.83×10^4 times that of ambient water thus exemplifying the ability of these algae to concentrate copper.

Analysis of *Macrocystis* juvenile sporophytes by x-ray fluorescence spectroscopy showed copper content to be 0.0063 μgat. Cu/g wet weight, a concentration ratio of 50.8 times that of seawater (North, 1977). In his analysis of mineral cycling in *Macrocystis*, Wort (1955) could find no specific pattern of maxima and minima for copper in the fronds and stipes of this kelp.

Sensitivities of benthic macroalgae to copper is a subject of increasing interest in the search for biological indicator species. Early work with *Macrocystis* includes the efforts of Clendenning and North (1960) to discover the effects of copper on the algal photosynthetic capacity. Measured manometrically, they found that the production of oxygen was inhibited 50% in 4 d by 100 ppb copper and that 60 ppb copper reduced photosynthesis 30% in 48 h.

Morris and Russel (1973) used *Ectocarpus siliculosus* as the test alga in a volumetric assessment of the growth retardant effects of various concentrations of copper up to 450 ppb. Watson (1976) found that concentrations of 14.5 ppb ionic copper (defined as that portion binding to Chelex-100 resin) resulted in the production of necrotic regions in the blades of *Ulva lobata* after 9 d exposure to copper-spiked medium.

The range in the copper concentrations that affect these macroalgae is seen to be quite large. This suggests that the form of the toxicant may be as important as the total concentration of metal to which the alga is exposed.

Copper Toxicity to *Macrocystis*

Copper toxicity in *Macrocystis* has not yet been fully investigated. This kelp is of major economic interest to California. The adult plants are harvested for their accumulated minerals to be used as food supplements and for the alginate that is extracted from the cell walls. Alginate is an important commercial polysaccharide, consisting of blocks of 1,4 linked D-mannuronic acid and L-glucuronic acid residues and alternating copolymer (Pigman, 1970; Seeley and Hart, 1974), that is used as an emulsifier and stabilizer in food and as a chelating agent and carrier in algicides and herbicides.

The living kelp forest is the habitat for many commercial marine organisms, including abalone and sport fishes. It serves as the source of primary production or energy base on which the rest of the kelp community—as well as the proximal sandy beach community—depends (Rosenthal, *et al.*, 1974). As a result of its importance, both directly and indirectly to the economy and ecology of California, we selected *Macrocystis* as the test alga.

Like all kelps, this alga reproduces by a heteromorphic alternation of generations: the diploid macroscopic stage alternates with a microscopic haploid phase (Fig. 1). *Macrocystis* reproduces continuously, but because reproductive blades seem to be especially susceptible to loss or damage in winter, periods of infertility exist in patches of the kelp forest. We used the early life stages of *Macrocystis*, spore and gametophyte, to test the toxicity of copper to this species. Previous experimental work with this alga suggests that, in the early life stages, there are certain "critical periods" during which the developing plant is more vulnerable to environmental stress (Gherardini and North, 1972). Each of the early stages of the alga were exposed to defined concentrations of copper and the toxic response was measured. For high copper concentrations, the toxic response was related to the total copper concentrations. For low copper concentrations, the toxic response was defined in terms of the speciation of the copper to which the plant had been exposed.

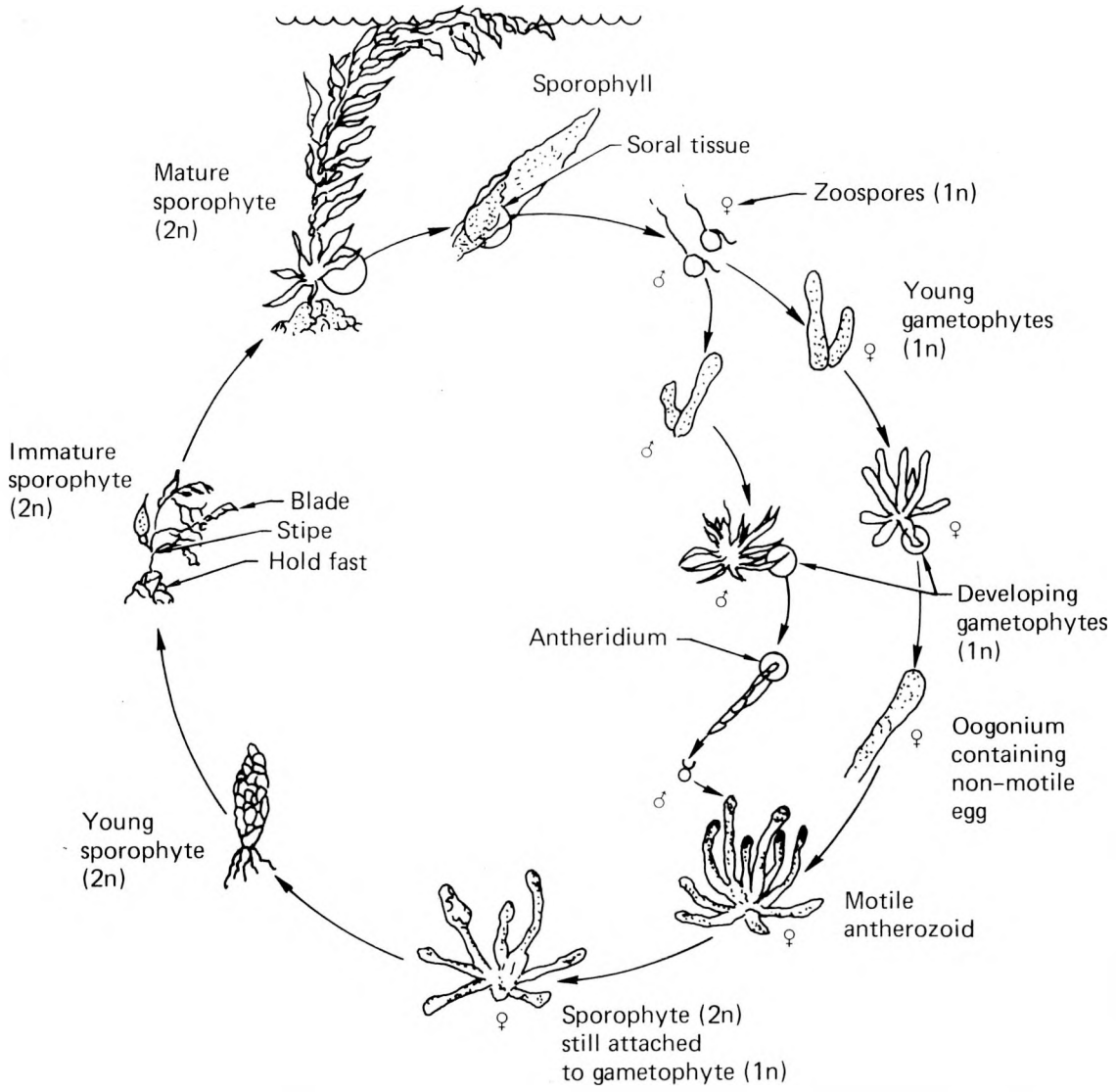


Fig. 1. The life cycle of *Macrocystis pyrifera*, showing the heteromorphic alternation of generations between the macroscopic diploid stage and the microscopic haploid stage.

MATERIALS AND METHODS

Bioassay System

The dish-culture unit consisted of a Nitex grid, punched with four holes, and a Teflon ring with corresponding holes that were slipped onto two glass rods in the bottom of a cylindrical culture vessel. The culture vessels were modified 70-by-100-mm glass algal storage jars. Two glass rods, approximately 1.5 cm long by 1 mm in diameter, were epoxied 2.75 in. apart on the inside bottom of the culture jars. Rings with a 3-in. o.d. and a 2.5-in. i.d. were cut from Teflon sheeting to weight down the Nitex grids. Four holes were punched into the ring at right angles to each other (see Fig. 2).

Culture vessels were suspended in the recirculating cooling seawater in Plexiglass "baskets" that fit into 70-gal aquaria (Fig. 3). The yokes of the baskets were attached to a variable-speed motor that gently shook the baskets during prescribed periods of the experiments. The vessels were not shaken during the first week of the experiments so that the settled spores could attach firmly to the Nitex substratum.

Each shaker unit was lighted continuously by two 4-ft, 40-W cool white fluourescent tubes that delivered an average of 1000 lux over the surface of the shaker basket. In experiments to determine the effects on sexual development, 3000 lux was supplied continuously after the first week to stimulate sexual maturation.

The grids onto which the spores were pipetted were prepared as follows. Nitex nylon netting (75-μm mesh) was cut into circles 3 in. in diameter. Four holes were cut at right angles to each other to fit onto the posts epoxied on the bottom of the culture vessels. The Nitex grid was washed repeatedly in redistilled water, blotted dry with paper toweling, and then arranged in a single layer in a plastic phototray before exposure to UV irradiation.* The washed grids were placed under a UV hood for 3 to 12 h per side, then examined under the microscope to ensure that no plant material remained. Clean, sterile grids were placed asceptically into individual sterile plastic petri dishes and stored in the UV hood.

The seawater used for these experiments was trucked in from Bodega Bay and stored in an underground reserve tank. Water from the reserve tank must be distinguished from that used for cooling the culture vessels and holding laboratory specimens before experimentation. The uncirculated seawater was filter-sterilized by pressurized flow through a porcelain cartridge filter with a pore size of less than 0.45 μm. The sterilized water was stored at room temperature, with aeration, in sterilized 5-gal Pyrex carbuoys until needed.

One litre of culture medium consisted of 980 ml of filter-sterilized seawater mixed asceptically with 16 ml of a stock phosphate/nitrate enrichment solution plus 4 ml of a stock trace metal solution. The phosphate/nitrate enrichment solution was made by dissolving 3.500 g $NaNO_3$, 0.628 g $NaHPO_4$, and 0.001 g KI in

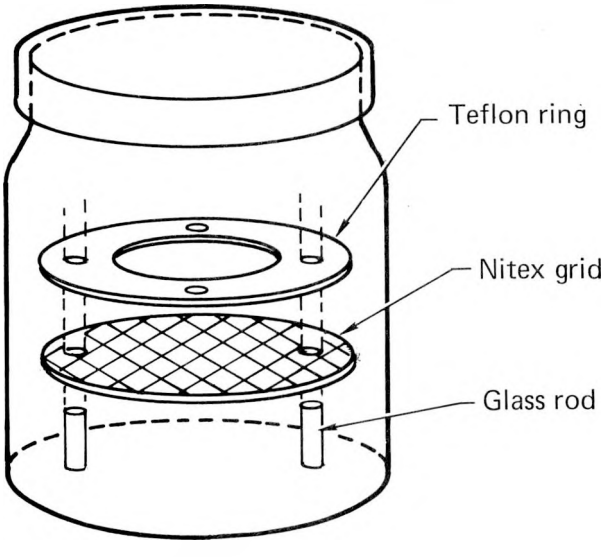


Fig. 2. Diagram of a culture vessel, showing the arrangement of the Nitex grid.

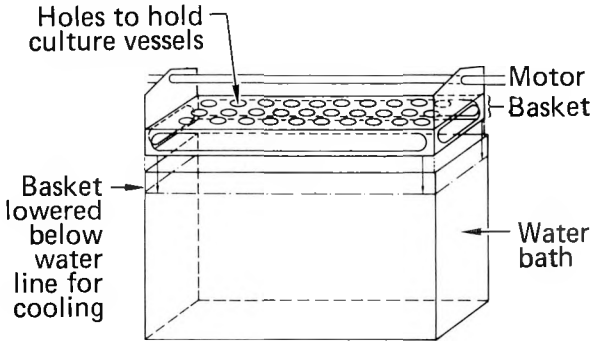


Fig. 3. Diagram of the shaker basket arrangement used to hold the culture vessels.

*Chlorox bleach was not used as a wash because it attacked the Nitex, making it brittle and rough. Ethyl alcohol was eliminated because it did no better than repeated redistilled water washes and because it increased the risk of organic contamination.

one litre of double-distilled water. The trace metal enrichment solution was made by dissolving 0.2859 g H_3BO_4, 0.0360 g $MnCl_2$, 0.0026 g $ZnCl_2$, and 0.0010 g $CoCl_2$ in 250 ml of double-distilled water. Then, the pH of both solutions was adjusted to 8.0, filter-sterilized, and refrigerated until needed. Originally, the trace metal mix recipe included Na_2EDTA and iron. Sodium-EDTA is a strong chelating agent that could have complexed the copper spikes as well as the iron which it was intended to keep in solution. Also, trace metals can be coprecipitated out of solution with iron. The amount of soluble iron in seawater was found to be less than the amount calculated to be present as an impurity in the salts of the trace metals mix (Byrne and Kester, 1976). Thus an iron deficiency was not expected to develop as a result of its exclusion from the trace metal enrichment solution. For these reasons, the Na_2EDTA and the iron were eliminated from the culture medium.

Copper used to spike the experimental medium was taken from a stock solution containing 5000 ppm copper (as copper chloride), dissolved in redistilled water, adjusted to a pH less than 2, and stored in a polyethylene bottle. This stock solution was used for all experiments; its concentration was verified on several occasions by flame atomic absorption spectrophotometry and differential pulse anodic stripping voltammetry.

Collection of Plant Material

Sporophyll blades of *Macrocystis pyrifera* were collected from the intake cove at the Diablo Canyon power plant. Blades taken from several plants at each collection were representative of the kelp population in the immediate area. If differences in sensitivity existed among individual kelp plants, this type of sampling procedure ensured a more representative batch of spores from the kelp population.

After collection, the plant material was rinsed with sterilized seawater to remove loosely attached ephiphytes. The individual blades were wrapped in paper toweling and placed in a plastic bag. The bag containing the blades was placed on ice in a styrofoam chest which was used for shipping. Several layers of plastic bags were placed between the ice and the blade-containing bag to prevent freezing. The ice chest was transported to LLL where the blades were used within 24 h of collection.

Laboratory Preparation

Ripe sporophylls were inspected for evidence of damage and only the intact blades (i.e., those with the fewest signs of breakage or grazing) were used. Intact blades were necessary because breakage leads to excessive mucus production which, in turn, encourages the growth of bacteria.

Healthy sporophylls were bent individually into sterilized, 100 or 150 ml beakers (Fig. 4). Filter-sterilized seawater was poured into the beakers to cover the blades. Several beakers of blades thus prepared were placed in a plastic phototray that was covered with aluminum foil to exclude light. The tray of beakers was refrigerated at 5°C for 1 h to trigger the release of spores.

After 1 h, the spore suspension was checked for its contents. In only one experiment was the spore content particularly low so that more than 1 h was required for adequate spore release. In earlier experiments when the spore suspension check revealed the presence of diatoms, the suspension was decanted, in the dark, into a 500-ml graduated cylinder that was wrapped in aluminum foil so that it was light-tight. The suspension was allowed to settle overnight at 5°C. In later experiments, the settling step was eliminated when we found that it did not change the diatom contamination.

The newly released spores are light sensitive and begin to settle within 5 minutes exposure to laboratory light conditions. If they were allowed to settle, a pipetting error might result. Therefore, we created a reduced-light work area

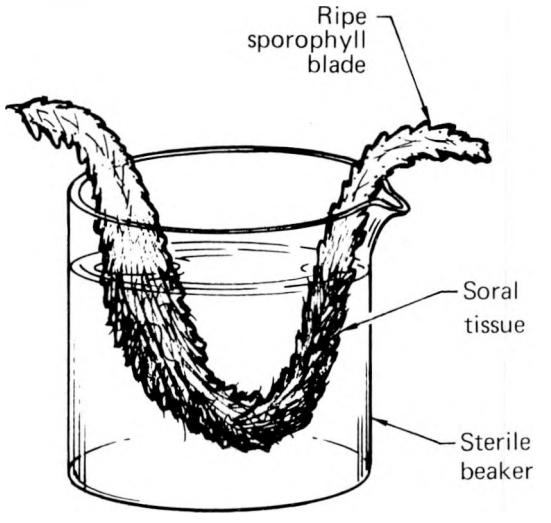


Fig. 4. Diagram showing a ripe sporophyll blade folded into a sterile beaker that will be refrigerated during the release of spores.

by draping a black cloth over a laboratory bench to eliminate light. A low-range light meter (0 to 1.5 ft-candles) registered zero inside the blacked-out work area.

Under the reduced-light conditions, a sterile pipette was used to take a small sample of the spore innoculum to be counted with a hemocytometer. This initial count was needed so that the spore innoculum could be adjusted to the desired concentration. A final count was made to check for a pipetting error resulting from spores settling on the walls of the graduated cylinder during the time the grids were being innoculated.

Disposable, sterile, plastic petri dishes containing the Nitex grids were arranged on an ice bath, in large plastic phototrays, in the "dark room." Spore innoculum was pipetted asceptically onto each grid, after which the trays were placed in light for 15 min. This settling period allowed the spores to attach to the Nitex grid.* The grids then were transferred to the culture vessels that contained the enriched culture medium, appropriately spiked with copper and cooled to the laboratory ambient water temperature of 12 ± 1°C.

Water Sample Preparation

The grids were placed in clean culture vessels with new culture medium each week. To obtain measurements of the average total copper in the medium to which the gametophytes actually had been exposed, the medium from all replicates of a given treatment or concentration level was pooled weekly. The pooled medium was stored temporarily in a polyethylene bottle, adjusted to pH 2 with concentrated HCl, until the total copper analysis could be performed.

Copper Analysis

After several experiments were concluded, total copper was analyzed in one of two ways depending on the initial spike concentration. For initial spikes of 100 ppb copper or more, total copper was analyzed with a Perkin Elmer Model 303 flame atomic absorption spectrophotometer. For initial spikes of less than 100 ppb copper, total copper was determined by flameless atomic absorption spectrophotometry using an HGA-2100 graphite furnace.

For experiments where the maximum concentration of copper spike was 50 ppb, differential pulse anodic stripping voltammetry (DPASV) also was used to analyze for both labile and total copper. This polarographic technique involves preconcentration of the metal from the solution to be analyzed by depositing it into the hanging drop mercury electrode (forming Cu°). This analysis is more sensitive than other voltammetric procedures because actual analysis is made by anodically stripping metal from the amalgam within the mercury drop. The concentration of the copper-mercury amalgam formed in the preconcentration step depends on the stirring rate of the test solution, the deposition time during which a current of known potential is applied to the working electrode, the radius of the drop of mercury, and the concentration of the metal in the solution (O'Dom and Siegerman, 1973). By standardizing the first three parameters for all analyses, the metal concentration in the test solution becomes the dependent variable.

Counting Procedure

Gametophyte growth was monitored weekly by a counting procedure developed to be consistent with the use of random statistics. Gametophytes grow by producing new filaments and by elongating existing ones. The resulting "bushy" growth pattern means that the percent cover methods or enumeration-type growth monitoring methods are inaccurate because these methods are unable to account for the three-dimensional aspects of growth. Therefore, we devised a variation of a line-intercept counting method. The number of filaments or "branches" that crossed the plane of a micrometer line, 450 μm long at 200× magnification, in each of 5 or 10 randomly placed fields on a grid was taken as the index of growth of the gametophytes on that grid (Fig. 5).

As the gametophyte grows, the chances that a filament will cross the line increase, both for filament production and for filament elongation. The drawbacks of this counting method are encountered in two situations. First, a grid with fewer gametophytes that grew more filaments is indistinguishable in count from one with more gametophytes that grew fewer filaments. Second, where gametophytes of one

*The preliminary settlement step was included because early attempts to pipette the spore innoculum directly into the culture vessels allowed a considerable portion of the developing gametophytes to attach to the sides of the culture vessel. Although settlement on the grids was random, the variation among grids was great enough to prohibit comparisons among treatments.

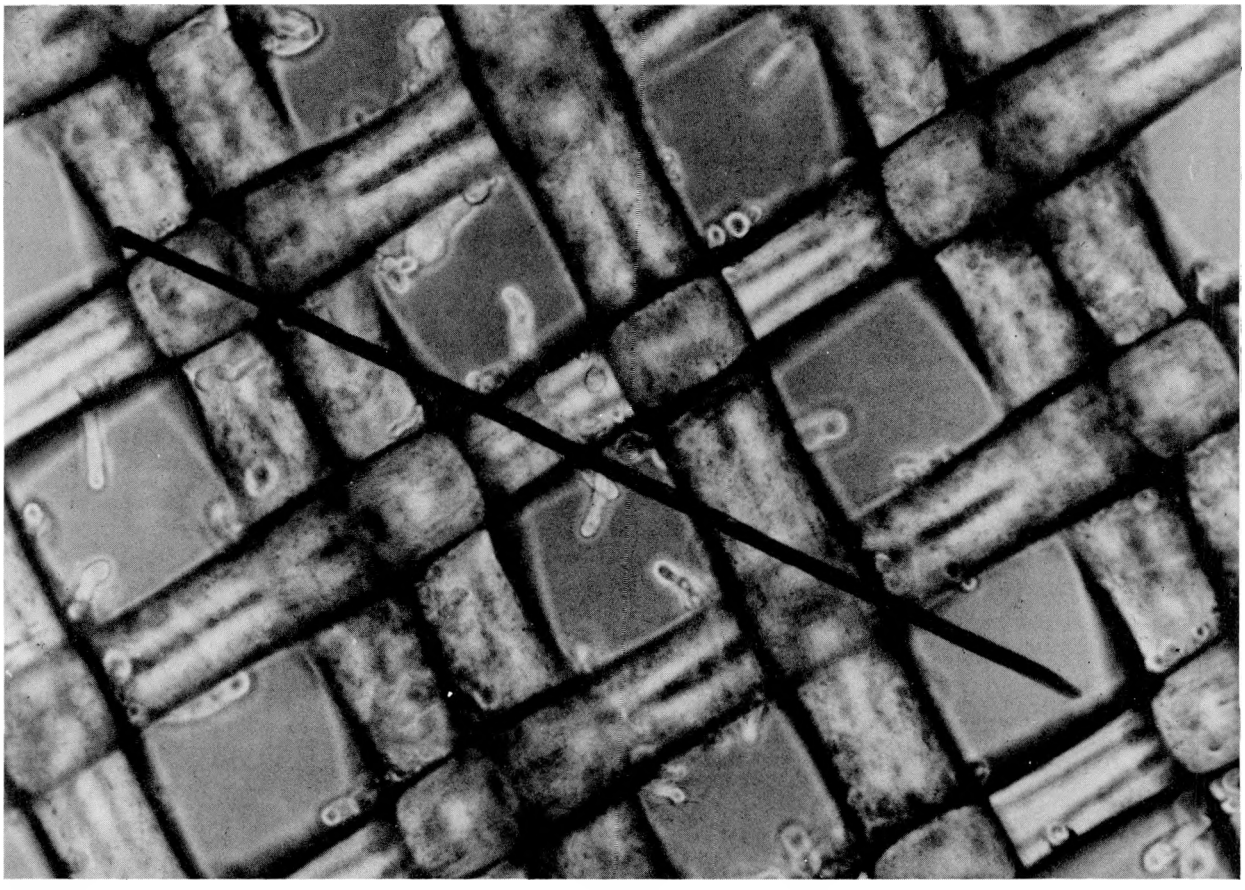


Fig. 5. Photomicrograph of the Nitex grid used to culture gametophytes, showing the relative sizes of the grid mesh and the micrometer line used for counting.

sex are differentially affected, the counting method would not detect this difference. The qualitative effects on growth that could not be monitored by this counting method were recorded with photomicrographs of the grids during different phases of some experiments.

Statistical Analysis

The graphs presented here were constructed with a plotter program designed for the Hewlett Packard Model 9820A calculator. The program computes the Chi Square values for three types of equations—straight line, power or exponential, and quadratic—and the user can select the type of equation that has the smallest Chi Square value. The program then plots the best-fit curve for those points according to the chosen type of equation.

The biological data was analyzed first by hand, then checked using a computer program that allowed the user to perform several different types of statistical tests. A single-factor analysis of variance (ANOV) was performed on the sum of the number of branches that crossed the micrometer line in all of the 5 or 10 randomly placed fields for each replicate grid to test for a difference among the mean values of the populations of copper-treated and untreated grids. Where the ANOV results indicate that the means of the populations were different, this test does not specify which among the means are different. The use of Dunnett's Test was a way of testing to see where the mean of the control differed significantly from that of each of the treatments. Because our experimental procedure was designed to compare the growth of spiked cultures with that of a control, Dunnett's Test was used in place of other types of multiple comparisons tests.

Additionally, we compared the growth of spiked cultures with that of controls and calculated the percent inhibition. The percent inhibition is defined as the mean number of branches that crosses the micrometer line in the control grid, minus the mean number of branches in the treated grid, divided by the mean of the control grid: $(\overline{X}_{control} - \overline{X}_{Cu})/\overline{X}_{control}$.

RESULTS

Types of Exposures

Several types of experiments were conducted to determine comprehensively the effects of copper on the growth of gametophytes of *Macrocystis pyrifera*. The first set of experiments involved the continuous exposure of gametophytes to various copper concentration levels. The second set was designed to determine the effects of copper exposure during particular stages of growth. The third type of experiments involved determining the time required for the gametophytes to recover from a pulsed exposure to copper.

Kelp can be exposed continuously to copper in environmental situations where the input varys only slightly over time, such as during the day-to-day operation of a power plant. This type of exposure generally will be to less than 10 ppb copper. Interspersed with continuous, low-range exposure, are periods of pulsed exposures to higher copper concentrations, such as during a shut-down in a power plant for routine maintenance. Temporary high-range copper exposure also could occur with the remobilization of metal-laden sediments resulting from storm action or bacterial metabolism. These types of environmental situations can be simulated in the laboratory with the use of continuous- and pulsed-exposure experiments.

The continuous-exposure experiments were divided into three categories, based on the concentrations of copper tested. Copper concentrations of 500, 1000, 2500, and 5000 ppb were categorized as high-range. Concentrations of 50 to 200 ppb copper were considered mid-range. The low-range category was composed of copper concentrations up to 50 ppb total copper. The medium was changed once a week in the high- and mid-range experiments and twice a week in the low-range experiments. The growth patterns of gametophytes exposed to high- and mid-range copper concentrations suggested that the copper in the medium was being detoxified at concentrations below 50 ppb; therefore, the ionic copper needed to be replaced more often at the lower levels.

Continuous High-Range Exposure

Experiments A and B were conducted with high concentrations of copper using two different concentrations of spore innoculum. Experiment A used a high spore concentration, whereas B used a low spore count.

Table 1 is a compilation of the data for Experiment A. After a 1-wk exposure to copper concentrations of greater than 500 ppb, gametophyte growth was inhibited 100%. After a 2-wk exposure to 200 ppb copper, growth was inhibited 80%, whereas 50 ppb copper suppressed growth 67% of that of the controls.

Table 2 shows the total copper remaining in the pooled water samples after a 1-wk exposure to the gametophytes. The percent recovery (defined as the total amount of the initial copper spike divided by the mean total copper measured in the water samples times 100) ranged from 58% (1000 ppb) to 96% (100 ppb), with an average of 75% for all concentrations in this experiment.

Table 3 presents the results of Experiment B where a low concentration of spore innoculum was exposed to high-range copper spikes. After a 2-wk exposure to 200 ppb copper and greater, growth was inhibited 100%; 50 ppb copper suppressed growth by 56%.

The results from the analyses of total copper in the pooled water samples are given in Table 4. The "recoveries" followed a pattern similar to the one seen for Experiment A. The percent recoveries ranged from 49% (1000

Table 1. High-range copper concentrations, Experiment A.

Copper concentration, ppb	Mean number of branches crossing micrometer line ± std. dev.		Percent inhibition	
	Week 1	Week 2	Week 1	Week 2
0 (control)	4.3 ± 2.9	29.0 ± 8.5	0	0
50	2.0 ± 1.4[a]	10.0 ± 2.8[b]	53	66
100	11.8 ± 2.2[b]	17.5 ± 5.3[b]	0	40
200	3.5 ± 1.9[a]	5.8 ± 2.9[b]	19	80
500	0.0[b]	0.0[b]	100	100
1000	0.0[b]	0.0[b]	100	100
2500	0.0[b]	0.0[b]	100	100
5000	0.0[b]	0.0[b]	100	100

[a] Growth not statistically different than controls.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

Table 2. Results of Experiment A chemical analyses.

Nominal copper concentration, ppb	Sum of number of branches crossing micrometer line (measured total copper, ppb)				Mean measured total copper, ppb ± std. dev.
	Week 1	Week 2	Week 3	Week 4	
0 (control)	17 (114)[a]	116 (80.2)[a]	214 (32.6)[a]	271 (26.2)[a]	–[c]
100	47 (96.3)	70 (115.2)	80 (65.2)	115 (106.1)	95.7 ± 21.8
200	14 (146.9)	23 (171.9)	21 (142.4)	49 (169.6)	157.7 ± 15.2
500	0 (336.3)	0 (476.0)	0 (294.4)	0 (310.3)	354.3 ± 83.0
1000	0 (609.5)	0 (557.7)	0 (–)[b]	0 (–)	583.6 ± 36.6
2500	0 (1648.7)	0 (1944.1)	0 (1313.3)	0 (–)	1635.4 ± 315.6
5000	0 (4034.0)	0 (3730.0)	0 (–)	0 (–)	3882.0 ± 214.96

[a] Storage containers were contaminated.
[b] Water sample was not analyzed.
[c] Mean for contaminated samples was not calculated.

ppb) to 81% (5000 ppb), with an average of 65% for all concentrations in this experiment.

The composite of the results of the high-range, continuous-exposure experiments (A and B) are presented in Table 5. For initial spikes of 500 ppb copper or greater, the percent copper recovery ranged between 52% (1000 ppb) and 79% (5000 ppb), with an average of 66% for all high-range concentrations of 500 ppb copper or more. The percent recoveries were 72% for 200 ppb and 100% for 100 ppb, with an average of 86% for these concentrations in Experiments A and B. The data indicate that, despite concentration-related differences, exposure to 500 ppb copper or greater resulted in 100% inhibition of the growth of the gametophyte within 1 wk. Exposures of 200 and 100 ppb copper depressed growth to approximately 85 and 60%, respectively, after a 4-wk period.

A graph for Experiment B, showing the relationship between growth (the total number of branches in five fields for all replicates of a given concentration) and time is given in Fig. 6. Exposure to more than 500 ppb copper is seen to result in total inhibition of gametophyte growth. Microscopic examination of the grids revealed that, after 1 wk, plant material deteriorated to the point of being unrecognizable.

Table 3. High-range copper concentrations, Experiment A.

Copper concentration, ppb	Mean number of branches crossing micrometer line ± std. dev.		Percent inhibition	
	Week 1[a]	Week 2	Week 1	Week 2
0 (control)	27.0 ± 14.9	19.5 ± 7.5	0	0
50	16.0 ± 2.8[b]	8.5 ± 3.5[c]	41	56
100	9.5 ± 5.7[c]	0.0[c]	65	100
200	6.8 ± 3.6[c]	0.0[c]	75	100
500	9.5 ± 5.7[c]	0.0[c]	65	100
1000	1.5 ± 1.0[c]	0.0[c]	94	100
2500	0.0[c]	0.0[c]	100	100
5000	0.0[c]	0.0[c]	100	100

[a] Used number of plants per field due to low spore concentration for Week 1 only.
[b] Growth not statistically different than controls.
[c] Growth differs significantly from controls (Dunnett's test, p = 0.05).

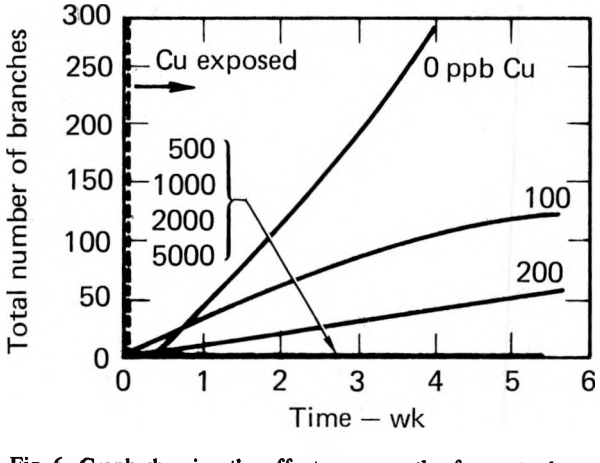


Fig. 6. Graph showing the effects on growth of gametophytes exposed to high-range copper concentrations vs time (Experiment B).

Table 4. Results of Experiment B chemical analyses.

Nominal copper concentration, ppb	Sum of number of branches crossing micrometer line (measured total copper, ppb)		Mean measured total copper copper, ppb ± std. dev.
	Week 1[a]	Week 2	
0 (control)	108 (41.5)[b]	78 (–)[c]	–[c]
100	38 (168.2)[b]	5 (120.1)[b]	–[d]
200	27 (158.7)	0 (135.8)	147.3 ± 16.2
500	38 (365.9)	0 (339.7)	352.8 ± 18.5
1000	6 (517.3)	0 (458.1)	487.7 ± 41.9
2500	0 (–)[c]	0 (1336.3)	1336.3
5000	0 (3918.3)	0 (4152.0)	4035.2 ± 165.3

[a] Used number of plants per field due to low spore concentration for Week 1 only.
[b] Storage container was contaminated.
[c] Water sample was not analyzed.
[d] Mean of contaminated samples was not calculated.

Table 5. Composite of results for high-range copper concentrations, Experiments A and B.

Nominal copper concentration, ppb	Mean measured total copper, ppb ± std. dev.	Percent inhibition of growth relative to controls			
		Week 1	Week 2	Week 3	Week 4
0 (control)	31.5 ± 7.4[a]	0	61	63	58
100	100.2 ± 23.7	0	61	63	58
200	142.6 ± 27.2	82	88	90	82
500	342.1 ± 67.5	100	100	100	100
1000	518.2 ± 82.1	100	100	100	100
2500	1635.4 ± 315.6	100	100	100	100
5000	3941.1 ± 170.8	100	100	100	100

[a] Storage containers were contaminated.

Continuous Mid-Range Exposure

Experiments also were performed to test the effects of continuous exposure to mid-range copper concentrations on the growth of gametophytes. From Table 6 we can see that two patterns emerge that are repeated throughout the experiments. First, as the initial spike of copper increases, the time to obvious growth retardation decreases. Thus in Experiment C, the growth of spiked cultures was statistically different from the controls after 2 wk of growth in medium spiked with 200 ppb copper, after 3 wk of growth in medium spiked with 100 ppb copper, and after 4 wk of growth in medium spiked with 50 ppb copper. The second emerging pattern is biomass-related. In Experiment C, the initial spore innoculum concentration was low and the time required for a given concentration of copper to alter growth to a statistically significant amount was shorter

Table 6. Mid-range copper concentrations, Experiments C and D.

Copper concentration, ppb	Time after which growth differs significantly from controls, wk	
	Experiment C	Experiment D
50	4	–
75	3	4
100	3	4
150	2	3
200	2	3

than in Experiment D, where the spore inoculum concentration was high. Thus for 200 ppb copper, the growth of spiked cultures was statistically different from controls after 2 wk in Experiment C (control mean = 7.75 branches; see Table 7), and 3 wk in Experiment D (control mean = 19.3 branches; see Table 8). In Experiment E (control mean = 5.8 branches, see Table 9), growth was statistically different at the p = 0.067 level after a 2-wk exposure to this concentration.

Tables 10, 11, and 12 present the results of the total copper analyses for Experiments C, D, and E, respectively. In Experiment C (Table 10), the percent recovery ranged from 60% (50 ppb) to 80% (25 ppb), with an average of approximately 70% for the mid-range concentrations as a whole. In Experiment D (Table 11), total copper analysis gave slightly higher values that probably were due to contamination of the storage containers. The percent recovery ranged from 76% (100 ppb) to 228% (25 ppb), with an average of approximately 80% for those analyses not obviously contaiminated (100, 150, and 200 ppb). In Experiment E (Table 12), the percent recovery ranged between 50% (50 ppb) and 77% (200 ppb), with an average of approximately 67% recovery for the mid-range concentrations as a whole. These recoveries were consistent with those calculated for initial spikes of greater than 200 ppb in the high-range experiments.

Table 7. Mid-range copper concentrations, Experiment C.

Copper concentration, ppb	Mean Number of Branches crossing micrometer line ± std. dev.			
	Week 1	Week 2	Week 3	Week 4
0 (control)	7.8 ± 2.1	13.5 ± 6.8	60.8 ± 9.9	114.0 ± 30.1
25	6.4 ± 3.5[a]	10.2 ± 3.4[a]	64.6 ± 17.8[a]	119.6 ± 14.8[a]
50	6.6 ± 3.9[a]	10.2 ± 4.0[a]	51.8 ± 20.8[a]	79.0 ± 21.9[b]
75	5.0 ± 1.4[a]	12.8 ± 1.0[a]	31.0 ± 11.5[a]	55.3 ± 11.6[b]
100	4.5 ± 2.4[a]	6.8 ± 4.3[a]	21.5 ± 7.4[b]	31.3 ± 22.1[b]
150	6.8 ± 2.9[a]	3.3 ± 4.0[a]	3.3 ± 1.5[b]	11.8 ± 3.4[b]
200	3.5 ± 3.1[a]	1.5 ± 1.7[b]	2.5 ± 1.0[b]	3.5 ± 2.5[b]

[a] Growth not statistically different than controls.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

Table 8. Mid-range copper concentrations, Experiment D.

Copper concentration, ppb	Mean number of branches crossing micrometer line ± std. dev.					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
0 (control)	19.3 ± 4.5	34.7 ± 6.7	46.7 ± 4.0	77.3 ± 5.9	102.3 ± 13.7	101.0 ± 2.6
25	13.7 ± 4.1[a]	37.3 ± 11.1[a]	49.0 ± 13.9[a]	80.3 ± 6.7[a]	99.0 ± 8.5[a]	95.7 ± 11.0[a]
50	23.7 ± 3.8[a]	41.7 ± 15.1[a]	63.7 ± 6.1[a]	71.0 ± 9.0[a]	89.7 ± 7.5[a]	85.7 ± 6.8[a]
75	25.0 ± 4.3[a]	43.3 ± 4.0[a]	58.7 ± 12.9[a]	49.3 ± 6.4[b]	54.3 ± 6.0[b]	44.0 ± 6.0[b]
100	18.3 ± 3.2[a]	31.3 ± 3.5[a]	29.0 ± 5.6[a]	23.3 ± 8.7[b]	26.7 ± 9.6[b]	23.7 ± 11.7[b]
150	22.0 ± 4.4[a]	23.7 ± 2.1[a]	21.0 ± 1.7[b]	17.0 ± 2.6[b]	19.3 ± 3.8[b]	17.3 ± 4.7[b]
200	16.7 ± 4.0[a]	17.0 ± 4.6[a]	12.3 ± 7.5[b]	9.0 ± 4.4[b]	7.0 ± 2.0[b]	4.3 ± 4.2[b]

[a] Growth not statistically different than controls.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

Table 9. Mid-range copper concentrations, Experiment E.

Copper concentration, ppb	Mean number of branches crossing micrometer line ± std. dev.				
	Week 1	Week 2	Week 3	Week 4	Week 5
0 (control)	5.8 ± 1.0	10.3 ± 5.4	38.0 ± 8.6	62.5 ± 5.1	86.5 ± 10.6
25	3.2 ± 1.3[a]	5.0 ± 2.2[a]	24.4 ± 6.9[b]	57.0 ± 10.3[a]	52.8 ± 6.8[b]
50	4.8 ± 2.8[a]	9.0 ± 2.4[a]	26.0 ± 4.8[b]	38.5 ± 5.2[b]	39.0 ± 4.1[b]
75	4.3 ± 1.9[a]	5.5 ± 3.7[a]	20.0 ± 3.7[b]	30.5 ± 5.8[b]	27.3 ± 8.2[b]
100	3.3 ± 0.5[a]	5.3 ± 2.6[a]	7.5 ± 4.7[b]	16.5 ± 10.5[b]	16.3 ± 7.1[b]
150	5.0 ± 0.8[a]	5.5 ± 1.3[a]	5.5 ± 2.9[b]	8.5 ± 2.6[b]	8.8 ± 3.9[b]
200	3.8 ± 2.2[a]	6.0 ± 2.9[a]	6.5 ± 5.3[b]	8.5 ± 4.4[b]	9.3 ± 7.4[b]

[a] Growth not statistically different than controls.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

Table 10. Results of Experiment C chemical analyses.

Nominal copper concentration, ppb	Sum of number of branches crossing micrometer line (measured total copper, ppb)					Mean measured total copper, ppb ± std. dev.
	Week 1	Week 2	Week 3	Week 4	Week 5	
0 (control)	31 (–)[a]	54 (6.2)	253 (3.3)	403 (5.0)	450 (2.0)	4.1 ± 1.9
25	29 (–)	42 (23.2)	270 (7.2)	339 (18.0)	494 (23.8)	18.1 ± 7.7
50	30 (–)	45 (20.1)	226 (12.6)	280 (34.0)	351 (29.8)	24.1 ± 9.6
75	20 (–)	47 (25.6)	126 (88.0)	216 (44.0)	221 (53.0)	52.7 ± 26.2
100	18 (–)	27 (23.3)	99 (87.0)	87 (71.0)	122 (92.0)	68.3 ± 31.3
150	27 (–)	13 (112.6)	13 (115.9)	19 (99.1)	47 (99.2)	106.6 ± 8.9
200	14 (–)	6 (178.3)	10 (158.6)	10 (141.9)	22 (135.4)	153.6 ± 19.2

[a] Water sample was not analyzed.

Table 11. Results of Experiment D chemical analyses.

Nominal copper concentration, ppb	Sum of number of branches crossing micrometer line (measured total copper, ppb)						Mean measured total copper, ppb
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
0 (control)	58 (7.8)	104 (–)[a]	140 (–)	232 (–)	307 (–)	303 (–)	7.8
25	41 (19.9)	112 (–)	147 (–)	241 (–)	297 (–)	267 (–)	19.9
75	75 (47.7)	130 (–)	176 (–)	148 (–)	163 (–)	133 (–)	47.7
100	67 (69.8)	96 (–)	95 (–)	70 (–)	80 (–)	71 (–)	69.8
150	66 (94.7)	71 (–)	61 (–)	51 (–)	58 (–)	52 (–)	94.7
200	50 (137.9)	50 (–)	37 (–)	27 (–)	21 (–)	13 (–)	137.9

[a] Water sample was not analyzed.

Table 12. Results of Experiment E chemical analyses.

Nominal copper concentration, ppb	Sum of number of branches crossing micrometer line (measured total copper, ppb)					Mean measured total copper, ppb ± std. dev.
	Week 1	Week 2	Week 3	Week 4	Week 5	
0 (control)	23 (–)[a]	41 (2.4)	152 (2.3)	260 (2.4)	343 (3.8)	2.7 ± 0.7
25	11 (–)	23 (61.7)	100 (56.0)	223 (–)	208 (53.7)	57.1 ± 4.1
50	19 (–)	36 (–)	104 (107.0)	154 (65.8)	156 (73.2)	82.0 ± 22.0
75	17 (–)	22 (82.8)	80 (99.9)	122 (84.8)	109 (90.7)	89.6 ± 7.7
100	13 (–)	21 (76.0)	30 (62.0)	66 (89.0)	107 (76.0)	75.8 ± 11.0
150	20 (–)	22 (100.0)	22 (110.0)	34 (139.0)	35 (134.0)	120.8 ± 18.8
200	15 (–)	24 (158.0)	26 (155.0)	34 (163.0)	37 (195.0)	167.8 ± 18.5

[a] Water sample was not analyzed.

Table 13 is a composite of the results of the mid-range copper concentration experiments. The mean values and standard deviations were calculated using all data regardless of the possibility of contamination. If all obviously contaminated samples are eliminated (those in which the measured total was larger than the initial spike), a pattern emerges that is consistent with the biological data. The percent recoveries for 25, 100, 150, and 200 ppb are close to 75%, whereas those for 50 and 75 ppb are 48 and 57%, respectively.

In the experiments with spikes of 150 and 200 ppb, growth was essentially halted from the time of initial exposure. Thus, the sum of the number of branches crossing the micrometer line—a rough index of algal biomass—was essentially unchanged throughout the exposure period for the 150 ppb (1.2) and the 200 ppb (1.0) copper concentrations. In contrast, for cultures exposed to 100 ppb copper, the sum tripled, and for 75 ppb copper, the sum of the number of branches was quadrupled (4.4) during the exposure period. For the exposure to 50 ppb copper, the sum of the number of branches increased nearly sixfold (5.7). The sum of the number of branches increased by a factor of 12.8 for gametophytes continuously exposed to 25 ppb copper and by a factor of 10.1 for control cultures.

Figure 7 illustrates the effects on gametophyte growth of copper concentrations between 25 and 200 ppb (Experiments C, D, and E). In each graph, the expected pattern appeared, increased copper in the medium resulted in decreased growth of the gametophytes. The biomass-related variation also appeared in the graphs that were drawn to the same scale: as the initial concentration of spore innoculum increased, each family of curves exhibited the inhibitory effects at a later period in development. Pooled data from Experiments C, D, and E is graphed in Fig. 8. After 1 wk, growth was inhibited 27% by 200 ppb copper and 10% by

Table 13. Composite of results for mid-range copper concentrations, Experiments C, D, and E.

Nominal copper concentration, ppb	Mean measured total copper, ppb ± std. dev.	Percent inhibition of growth relative to controls				
		Week 1	Week 2	Week 3	Week 4	Week 5
0 (control)	3.9 ± 2.0	0	0	0	0	0
25	32.9 ± 20.8	28	11	5	10	9
50	46.5 ± 32.4	0	0	3	28	31
75	63.6 ± 22.7	0	0	30	44	55
100	71.8 ± 20.7	10	28	59	75	72
150	111.6 ± 15.8	0	47	82	88	87
200	158.1 ± 19.3	28	60	87	92	93

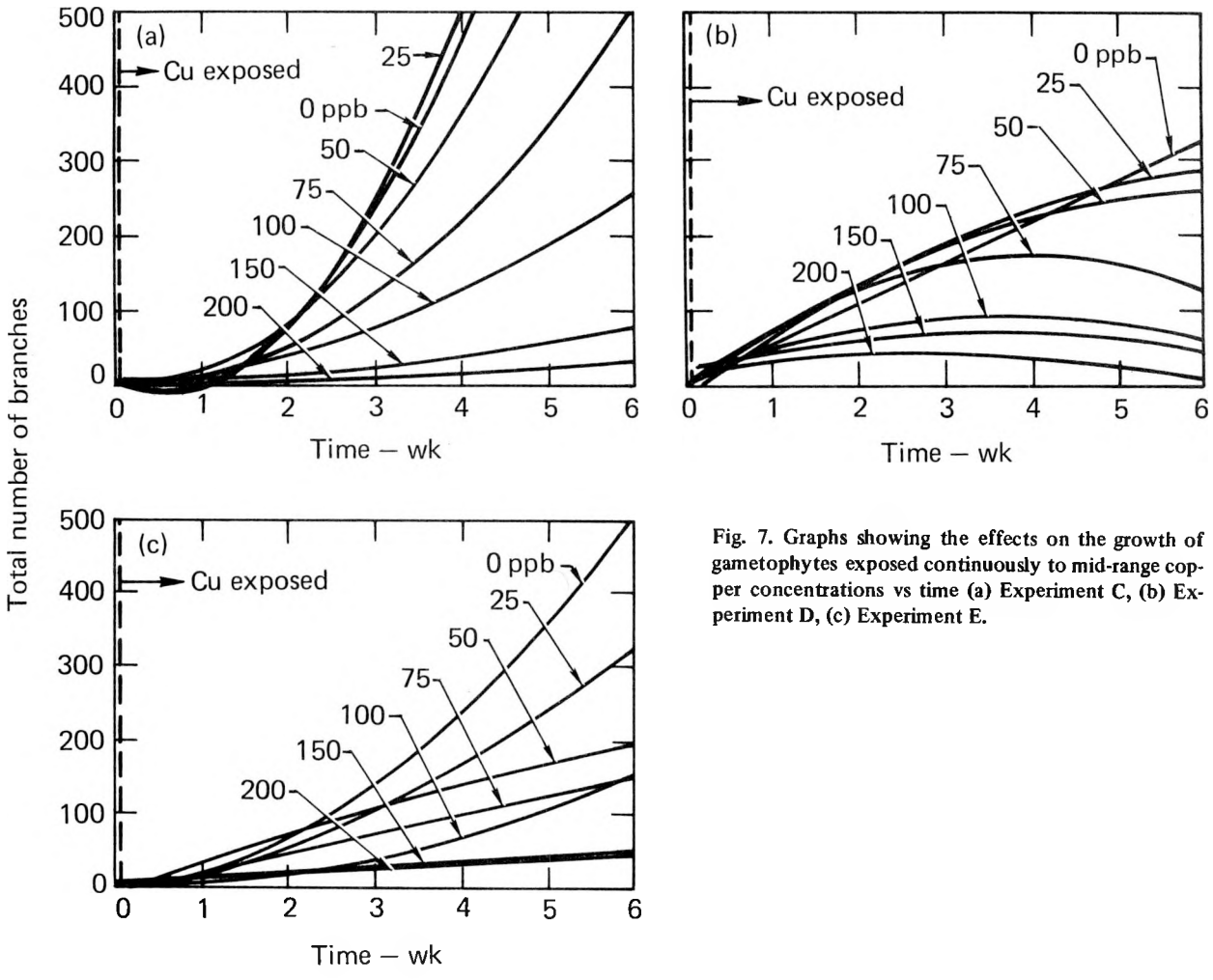


Fig. 7. Graphs showing the effects on the growth of gametophytes exposed continuously to mid-range copper concentrations vs time (a) Experiment C, (b) Experiment D, (c) Experiment E.

100 ppb copper. After 2 wk of continuous exposure to 100 ppb copper, growth was depressed 27%, and 150 ppb copper resulted in a growth suppression of 47%. After 5 wk, cultures spiked with 50 ppb copper showed a 31% inhibition of growth, a 100-ppb copper spike resulted in 72% growth inhibition, a 150-ppb copper spike resulted in 87% growth reduction, and a 200-ppb copper spike resulted in 93% inhibition of growth compared to that of the controls (Table 13). The growth of cultures spiked with 25 ppb copper was not statistically different in Experiment C after a 5-wk exposure, although this period of exposure was sufficient to cause growth retardation in Experiments D and E. The pooled results indicate a 9% growth inhibition for the three experiments at this concentration.

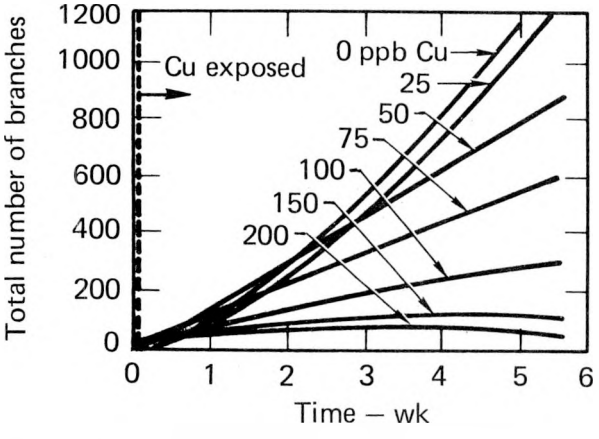


Fig. 8. Graph showing the effects on the growth of gametophytes exposed continuously to mid-range copper concentrations vs time for data pooled from Experiments C, D, and E.

The photomicrographs of the grids of Experiment D recorded the physical appearance of control and treated cultures. A photograph of a control culture after 6 wk of growth is shown in Fig. 9a and the effect of gametophyte exposure to 50 ppb copper is shown in Fig. 9b. The copper-exposed gametophytes were stubbier in appearance than the controls and lacked

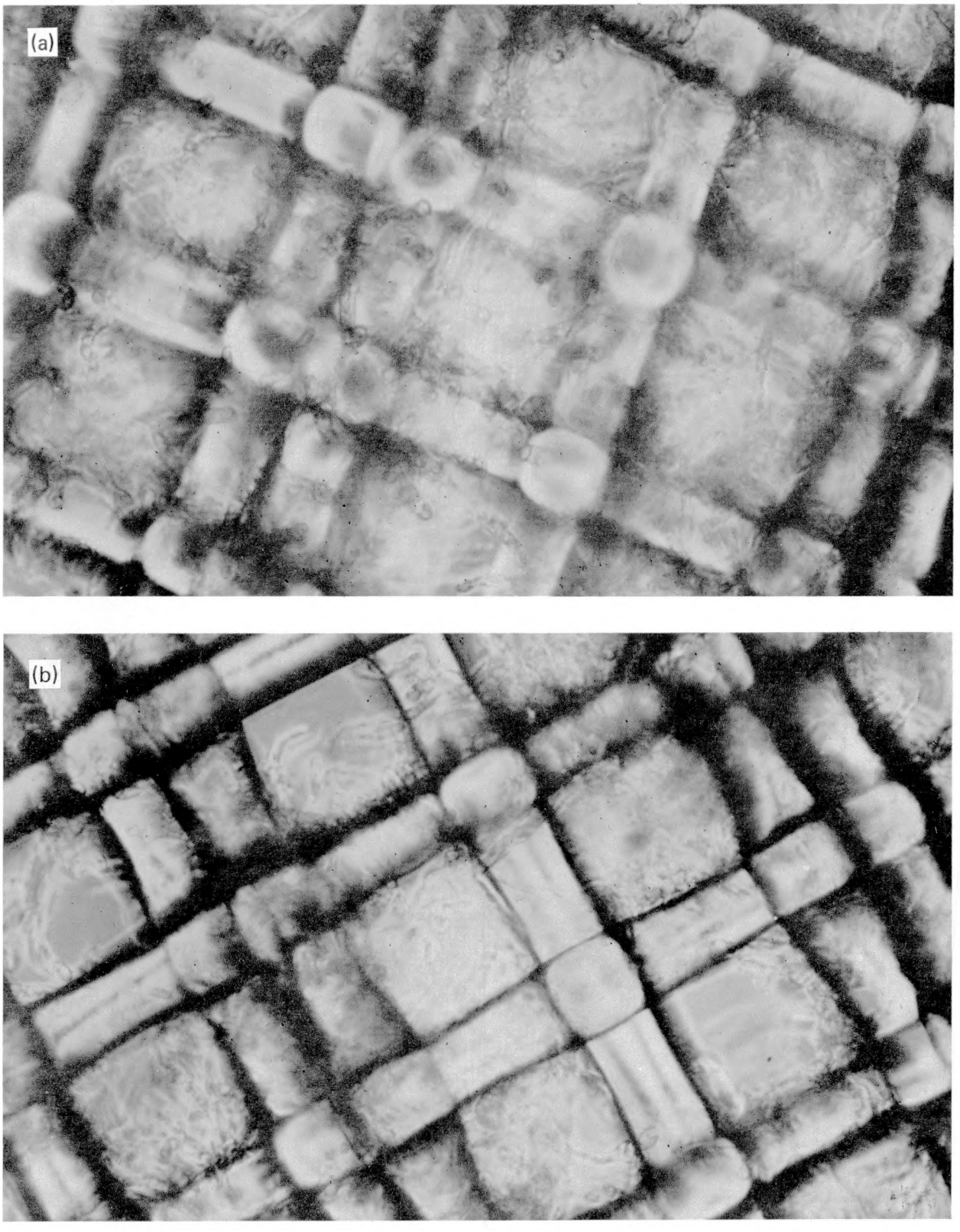
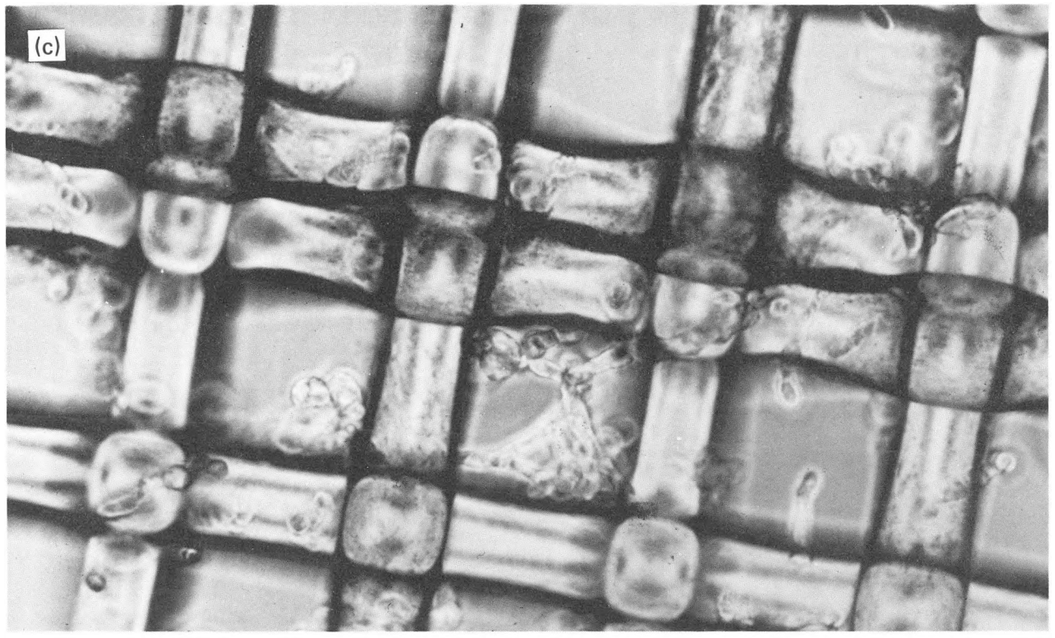
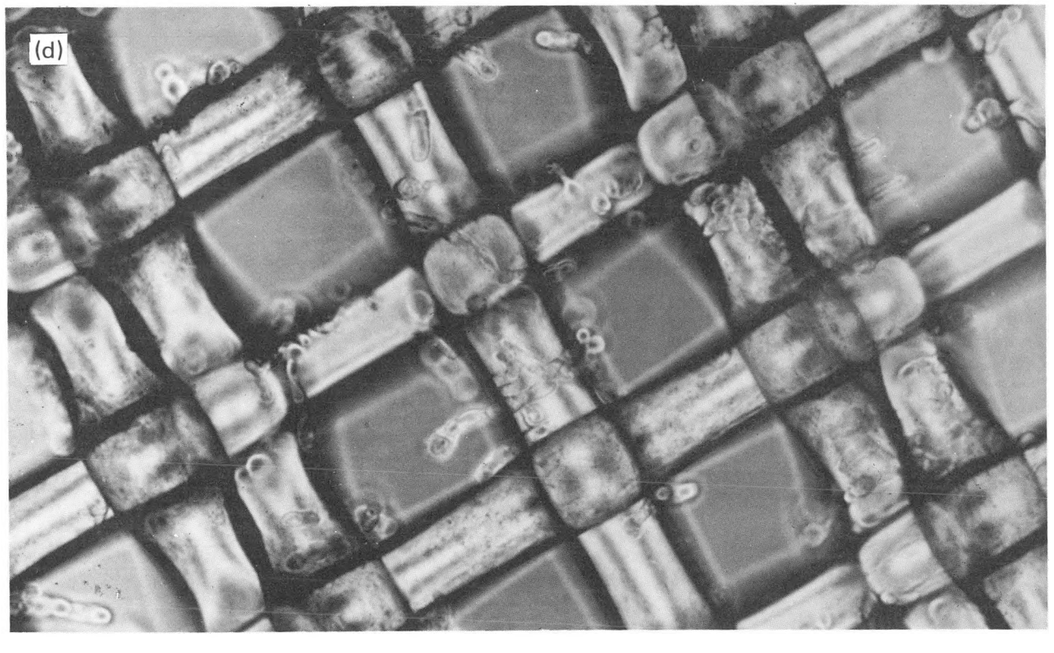
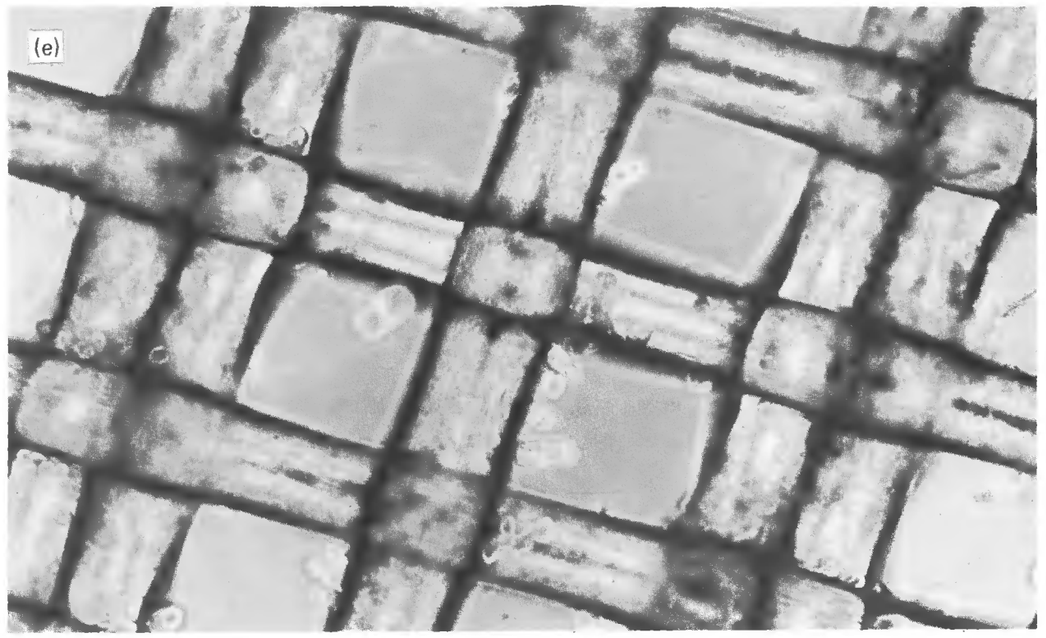


Fig. 9. Photomicrographs of a 6-wk-old control culture of *Macrocystis* gametophytes (a), and of 6-wk-old cultures of gametophytes exposed continuously to (b) 50 ppb copper.







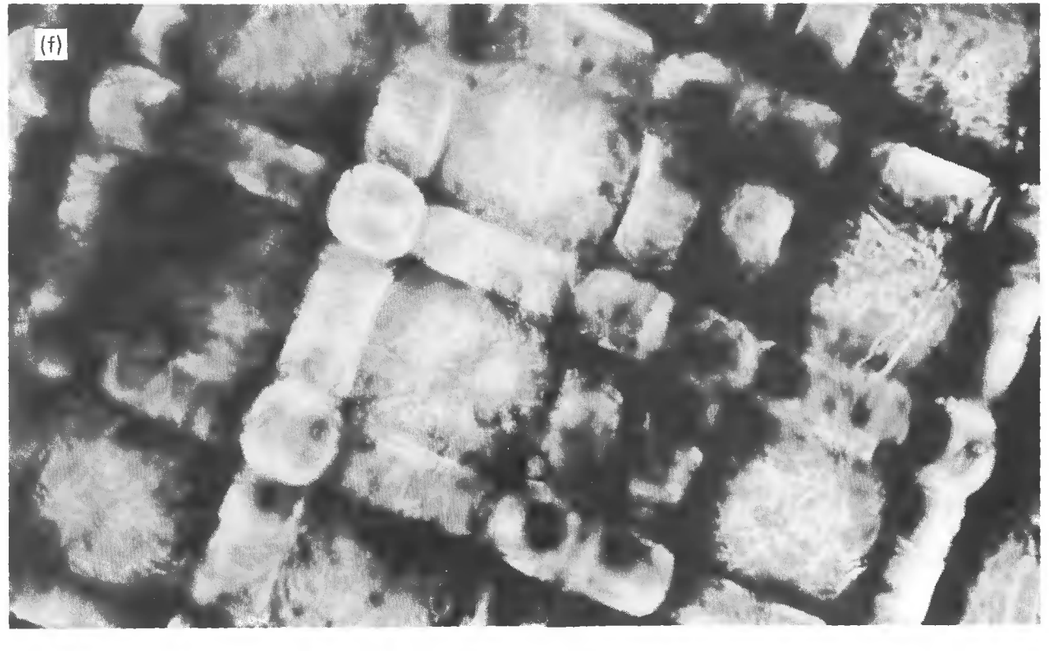


Fig. 9. 6-wk-old cultures of gametophytes exposed continuously to (c) 100 ppb copper, (d) 150 ppb copper, (e) 200 ppb copper, and (f) 25 ppb copper.

the distinctive sexual characteristics of the control plants. Photomicrographs showing the effects of 6 wk of exposure to 100, 150, and 200 ppb copper are given in Figs. 9c–e, respectively. The treated cultures show a growth pattern characterized by fewer, shorter filaments that seem to branch randomly. At 200 ppb copper, the plants exposed for 6 wk did not seem to have progressed past the growth level that would have been reached by some control cultures after 1 or 2 wk.

The appearance of a 6-wk-old culture spiked with 25 ppb copper is shown in Fig. 9f. The gametophytes appear "normal," in the sense that there is some differentiation between sexual types, although the distinctions are harder to draw in the treated cultures. The number of filaments produced by the gametophytes of cultures spiked with less than 50 ppb copper seems to be roughly similar to the controls. However, in these cultures the "viability" of the treated gametophyte, i.e., the ability of the gametophyte to produce sexual products (eggs and antherozoids), does differ.

Continuous Low-Range Exposure

Four experiments were conducted involving continuous exposure of gametophytes to low-range copper concentrations ($\leqslant$ 50 ppb). In the low-range experiments, the light regime was changed to deliver 3000 lux continuously after the first week of growth so that the gametophytes may have been growing at a faster rate, making any growth inhibition more obvious in a shorter period of time.

Table 14 presents the biological data for paired Experiments F and G. Experiment F was innoculated with half the concentration of spore suspension as used in Experiment G. In Experiment F, exposure to copper concentrations of less than 50 ppb had no statistically significant effect on growth, although a 2-wk exposure to 50 ppb copper reduced the mean number of branches by 17%. In Experiment G, there was a statistically significant difference in the growth of cultures spiked with 30 and 50 ppb copper after 2 wk of exposure to these concentrations.

Table 15 shows the mean number of branches for paired Experiments H and I. As in the previous two experiments, Experiment H was innoculated with half the concentration of spore suspension as used in Experiment I. Graphs of Experiments H and I are presented in Fig. 10. Initial spikes of 20 ppb copper or less had little or no effect on the growth of the gametophyte, although spikes of 30 and 50 ppb copper depressed growth demonstrably.

Table 14. Low-range copper concentrations, Experiments F and G.

Copper concentration, ppb	Mean number of branches crossing micrometer line ± std. dev. (percent inhibition)			
	Experiment F		Experiment G	
	Week 1	Week 2	Week 1	Week 2
0 (control)	13.8 ± 3.5 (0)	16.3 ± 4.2 (0)	19.6 ± 5.4 (0)	21.2 ± 5.3 (0)
10	11.9 ± 2.8[a] (14)	17.8 ± 3.3[a] (0)	20.3 ± 3.2[a] (0)	16.9 ± 2.7[b] (20)
20	12.8 ± 2.9[a] (7)	16.1 ± 3.6[a] (1)	21.1 ± 6.6[a] (0)	18.5 ± 2.2[a] (13)
30	13.1 ± 3.5[a] (5)	18.2 ± 3.9[a] (0)	22.1 ± 8.0[a] (0)	14.5 ± 3.5[b] (32)
50	15.1 ± 4.9[a] (0)	13.5 ± 4.8[a] (17)	23.8 ± 3.4[a] (0)	13.7 ± 3.0[b] (35)

[a] Growth not statistically different than controls.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

Table 16 is a composite of the results of Experiments F, G, H, and I. The average percent inhibition of growth after 2 wk of exposure is 12% for the 10-ppb spike, 10% for the 20-ppb spike, 21% for the 30-ppb spike, and 28% for the 50-ppb initial copper spike. These values were somewhat higher than those obtained for the 25- and 50-ppb spikes in the mid-range experiments.

Table 16 also presents the results from the DPASV analyses of the test media for low-range copper exposures. The gametophytes have a profound effect on the medium: not only do they complex the copper, they also adsorb it. The amount of copper adsorbed by the surfaces of the culture vessel was estimated by measuring the total copper in the media that had been stored for 1 wk in culture vessels without algae. For nominal concentrations of 10, 20, and 30 ppb copper, the recoveries were 86 to 100%. Thus, the "loss" of copper attributable to adsorption onto the culture vessel and to formation of nonoxidizable copper complexes, is about 15% of the total spike. For 50

Table 15. Low-range copper concentrations, Experiments H and I.

Copper concentration, ppb	Mean number of branches crossing micrometer line ± std. dev.					
	Experiment H			Experiment I		
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
0 (control)	4.5 ± 1.5	11.3 ± 3.0	12.8 ± 3.7	1.9 ± 0.9	7.3 ± 1.8	15.4 ± 3.9
10	3.9 ± 0.8[a]	8.0 ± 1.6[b]	15.7 ± 3.2[a]	2.5 ± 1.4[a]	9.7 ± 2.7[b]	13.7 ± 3.2[a]
20	4.8 ± 1.7[a]	9.7 ± 2.7[a]	15.3 ± 1.8[a]	2.3 ± 1.0[a]	6.4 ± 2.4[a]	8.8 ± 3.2[b]
30	4.8 ± 1.7[a]	8.0 ± 2.7[b]	9.7 ± 2.0[b]	2.3 ± 1.0[a]	6.5 ± 1.5[a]	8.6 ± 2.1[b]
50	3.6 ± 1.1[a]	6.6 ± 1.9[b]	9.6 ± 2.3[b]	1.9 ± 0.9[a]	3.6 ± 1.7[b]	4.5 ± 1.5[b]

[a] Growth not statistically different than controls.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

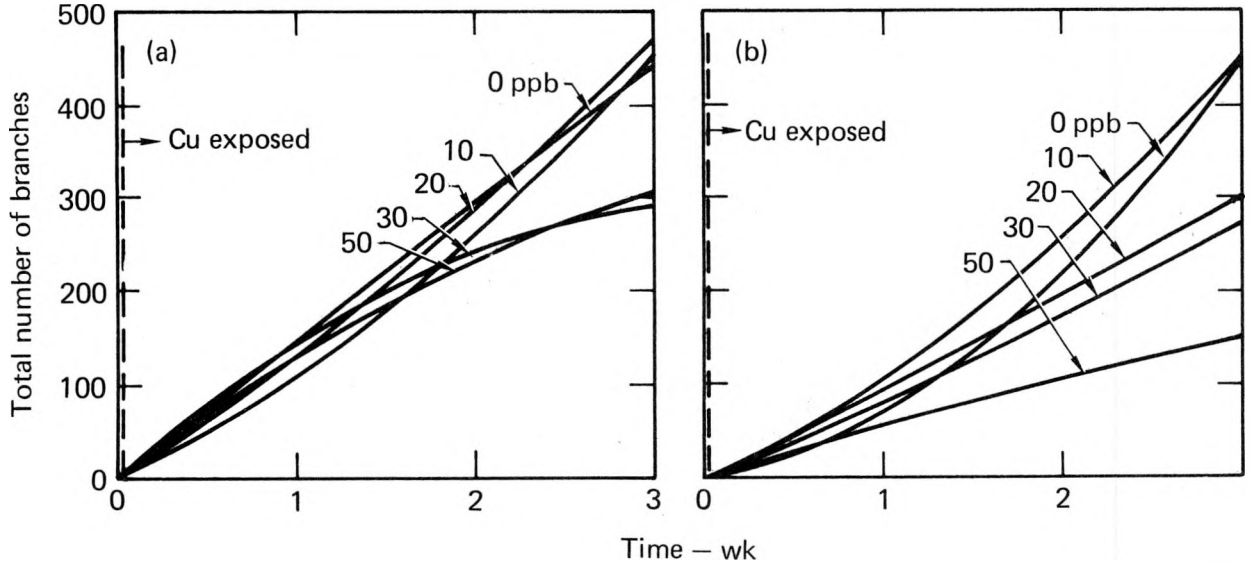


Fig. 10. Graphs showing the effects on the growth of gametophytes exposed continuously to low-range copper concentrations vs time for Experiment H (a) and for Experiment I (b).

Table 16. Composite of results for low-range copper concentrations, Experiments F, G, H, and I chemical analyses.

Nominal copper concentration, ppb	Mean percent inhibition of growth relative to controls (range)			DPASV mean measured copper, ppb ± std. dev.	
	Week 1	Week 2	Week 3	Total	Labile
0 (control)	0	0	0	3.4 ± 2.6[a] (2.6 ± 0.16)[b]	1.1 ± 1.8 (0.9 ± 0.1)
10	4 (0–14)	12 (0–36)	0	10.8 ± 7.3 (7.2 ± 2.4)	4.5 ± 4.9 (2.9 ± 2.1)
20	2 (0–8)	10 (2–28)	17 (0–34)	17.4 ± 11.4 (9.2 ± 3.6)	11.3 ± 10.9 (0.4)
30	2 (0–9)	21 (0–46)	34 (30–39)	25.7 ± 9.5 (13.0 ± 5.7)	20.2 ± 11.0 (1.2)
50	7 (0–12)	28 (0–52)	48 (29–66)	47.6 ± 0.2 (14.9 ± 4.3)	33.8 ± 1.8 (0.6)

[a]Measured in media without algae.
[b]Measured in media containing algae.

ppb copper in culture vessels without algae, the percent recovery rose to 95%, although the absolute amount of copper lost to the culture vessels remained in the 3-to-5-ppb range.

The question of how much copper was available to the plant, i.e., how much the plant "sees," was estimated by DPASV analysis of the amount of ASV-labile copper that was present in the medium. The effect of the culture vessel on copper lability was also determined by analyzing media that had been stored for 1 wk in vessels without algae. Of the approximately 11-ppb mean total copper measured for a 10-ppb spike, 4.5 ppb was estimated to be labile (Table 16); of the 17-ppb mean total copper recorded for a 20-ppb spike, an average of 11 ppb was labile. In addition, for a spike of 30 ppb copper, about 26 ppb could be recovered, of which 20 ppb was calculated to be labile; for a 50-ppb spike, 48 ppb copper was detectable, of which 34 ppb was ASV-labile.

The ASV-labile copper content of algae-exposed medium (Table 16) was determined as above and found to range from 0.4 ppb (20 ppb) to 2.9 ppb (10 ppb), with an average of 1 ppb over all concentrations in this low range.

Exposure of 1-wk-old Gametophytes

The second type of experiment was conducted to determine the effects of copper on various stages of gametophyte development. High-range exposures were not performed because the algal material disintegrated after a 1-wk exposure at these copper levels. Gametophytes at three stages of development were exposed to mid-range copper concentrations from 50 to 200 ppb. Water samples were not collected for these experiments.

Table 17 shows the results of exposure of 1-wk-old gametophytes to various concentrations of copper (Experiment J). After a 1-wk exposure to 150 and 200 ppb copper, growth of the spiked cultures was statistically different than that of controls.

After 2 wk of exposure, the growth of cultures spiked with 50 and 100 ppb copper was significantly depressed when compared to that of controls. A graph of the total number of branches crossing the line in each of five fields for three replicates vs time is shown in Fig. 11a. The growth of the gametophytes was essentially halted after a 1-wk exposure to concentrations greater than 100 ppb copper.

Exposure of 2-wk-old Gametophytes

The biological data for Experiment K involving exposure of 2-wk-old gametophytes to mid-range copper concentrations is presented in Table 18. After a 1-wk exposure to 100 and 200 ppb copper, the growth of the spiked cultures was statistically different than that of the controls. A graph of the total number of

Table 17. Results of mid-range copper concentrations of Experiment J: Exposure of one-week-old gametophytes.

Copper concentration, ppb	Mean number of branches crossing micrometer line ± std. dev. (percent inhibition)				
	Week 1	Week 2	Week 3	Week 4	Week 5
0 (control)	20.0 ± 2.0 (0)	43.7 ± 3.1 (0)	67.3 ± 3.5 (0)	84.3 ± 14.0 (0)	89.7 ± 6.7 (0)
50	19.0 ± 1.0[a] (5)	42.0 ± 8.9[a] (4)	37.3 ± 15.0[b] (45)	41.7 ± 8.1[b] (51)	48.7 ± 8.3[b] (46)
100	18.7 ± 3.8[a] (7)	33.7 ± 4.5[a] (23)	39.3 ± 8.5[b] (42)	36.0 ± 7.8[b] (57)	29.0 ± 2.6[b] (68)
150	18.3 ± 5.0[a] (9)	27.3 ± 7.6[b] (38)	29.3 ± 2.3[b] (56)	22.7 ± 9.1[b] (73)	18.7 ± 2.3[b] (79)
200	17.7 ± 6.4[a] (12)	22.0 ± 7.0[a] (50)	20.0 ± 4.4[b] (70)	14.0 ± 2.6[b] (83)	7.7 ± 2.9[b] (91)

[a] Growth not statistically different than controls.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

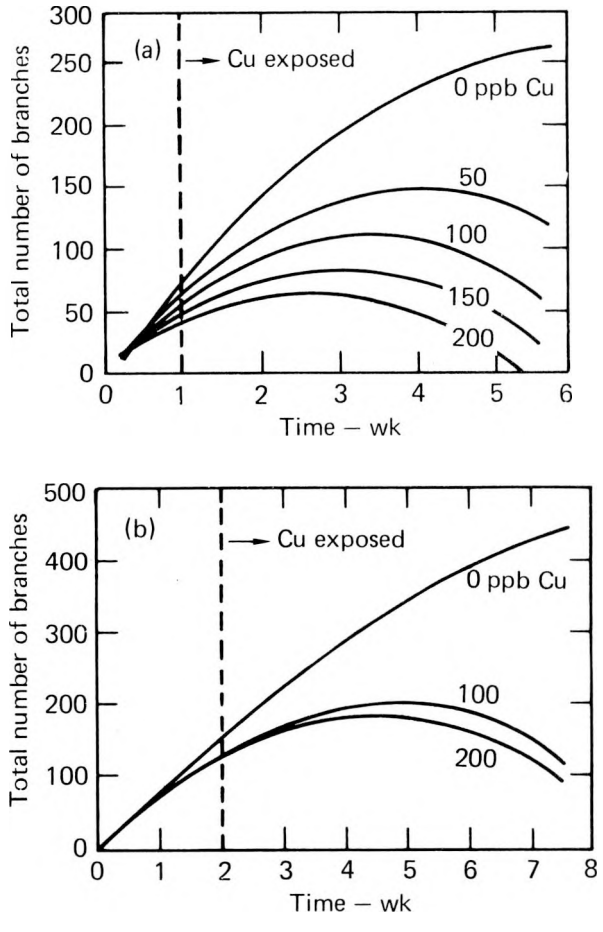


Fig. 11. Graphs showing the effects on the growth of (a) 1-wk-old gametophytes (Experiment J) and (b) 2-wk-old gametophytes (Experiment K) exposed continuously to mid-range copper concentrations vs time.

branches vs time is given in Fig. 11b. As in the previous experiment, growth of the copper-exposed cultures was essentially halted during a 1-wk exposure to copper spikes of greater than 100 ppb. A photomicrograph of a control culture from this experiment taken after 6 wk of growth is shown in Fig. 12a, the results of a 4-wk exposure to 100 ppb are given in Fig. 12b, and Fig. 12c shows the condition of the cultures exposed to 200 ppb for 4 wk in this experiment. In Fig. 12c, the size of the copper-exposed 6-wk-old gametophytes, in terms of the number of filaments, is seen to be roughly similar to those of 2-wk-old control cultures.

Exposure of 5-wk-old Gametophytes

Table 19 shows the results of continuous exposure of 5-wk-old gametophytes to mid-range copper concentrations. After a 1-wk exposure to a copper spike of 200 ppb, the growth of these cultures was statistically different from that of the controls. After 2 wk of exposure to 200 ppb copper, the growth of the spiked cultures differed significantly from that of the controls. Figure 13, a graph of the total number of branches crossing the micrometer line in five fields for all of three replicates vs time, shows that the growth of the gametophyte was essentially halted after a 1-wk exposure to 200 ppb copper.

Table 18. Results of mid-range copper concentrations of Experiment K: Exposure of two-week-old gametophytes.

Copper concentration, ppb[a]	Mean number of branches crossing micrometer line ± std. dev. (percent inhibition)				
	Week 3	Week 4	Week 5	Week 6	Week 7
0 (control)	86.0 ± 9.2 (0)	99.0 ± 6.1 (0)	110.3 ± 8.4 (0)	123.0 ± 10.6 (0)	152.7 ± 5.5 (0)
100	65.3 ± 10.0[b] (24)	59.0 ± 11.1[b] (40)	63.0 ± 16.7[b] (43)	69.0 ± 4.0[b] (44)	51.3 ± 5.1[b] (66)
200	53.0 ± 10.2[b] (38)	51.7 ± 12.0[b] (48)	66.3 ± 12.5[b] (40)	58.3 ± 13.6[b] (53)	41.0 ± 7.0[b] (73)

[a] Water samples not taken for this experiment.
[b] Growth differs significantly from controls (Dunnett's test, p = 0.05).

Table 19: Results of mid-range copper concentrations, Experiment L: Exposure of five-week-old gametophytes.

Copper concentration, ppb[a]	Mean number of branches crossing micrometer line ± std. dev. (percent inhibition)		
	Week 6	Week 7	Week 8[b]
0 (control)	123.0 ± 10.6 (0)	152.7 ± 5.5 (0)	84.0 ± 2.7 (0)
100	115.3 ± 4.0[c] (6)	122.3 ± 7.5[d] (20)	68.3 ± 5.9[d] (19)
200	87.0 ± 8.9[d] (29)	100.7 ± 7.2[d] (34)	45.7 ± 6.8[d] (46)

[a] Water samples not taken for this experiment.
[b] Counted three fields for Week 8 only.
[c] Growth not statistically different than controls.
[d] Growth differs significantly from controls (Dunnett's test, p = 0.05).

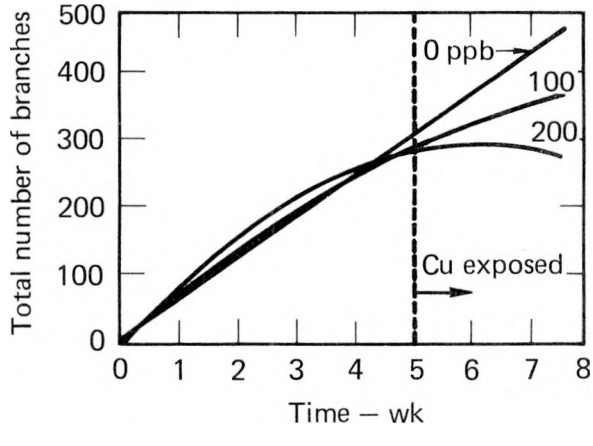
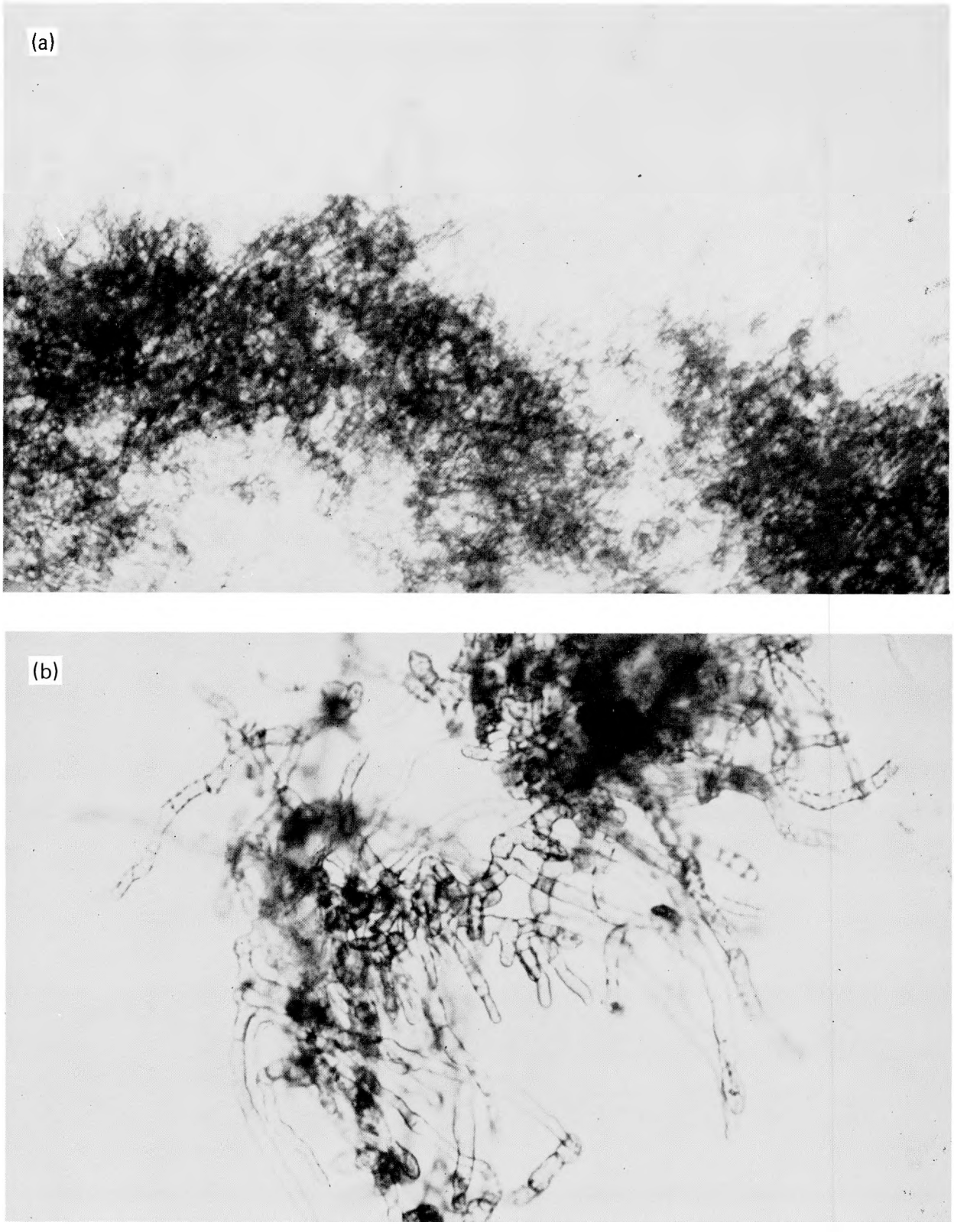


Fig. 13. Graph showing the effects on the growth of 5-wk-old *Macrocystis* gametophytes exposed continuously to mid-range copper concentrations vs time (Experiment L).


(a)
(b)

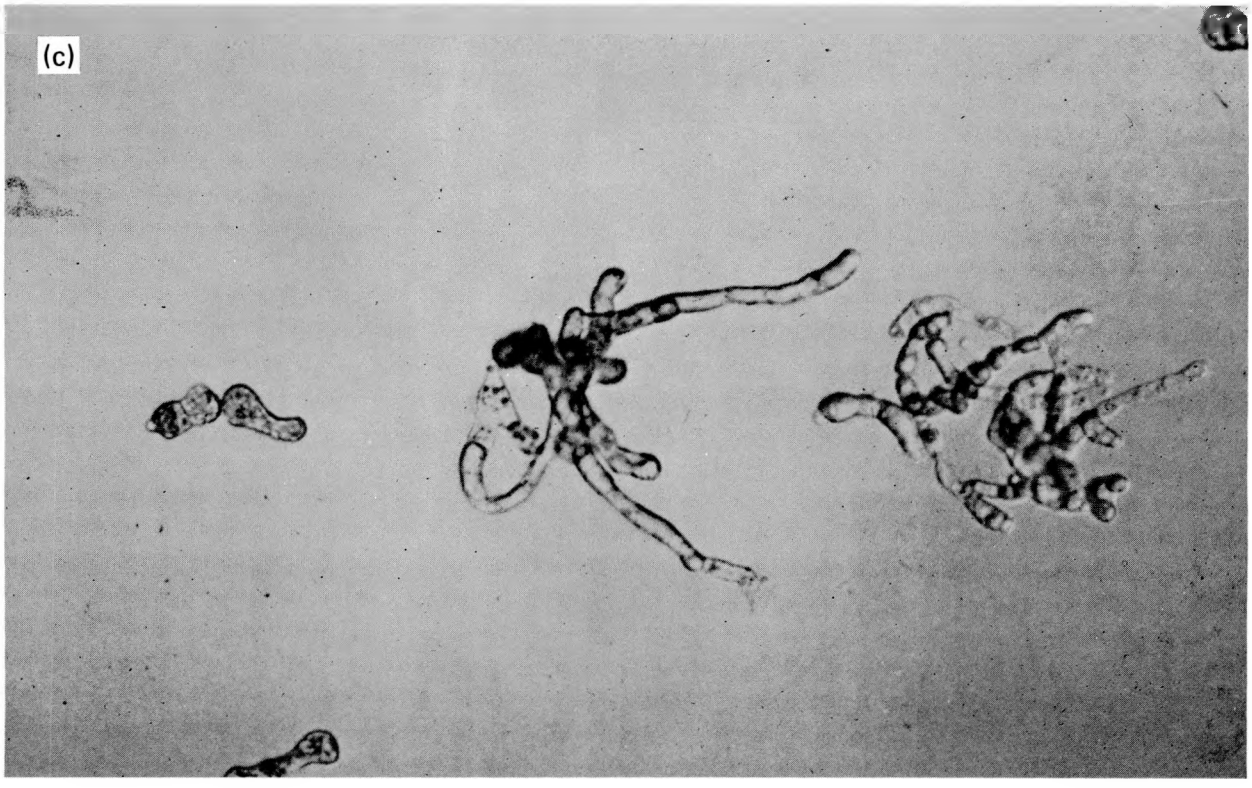


Fig. 12. Photomicrographs of a 6-wk-old control culture of *Macrocystis* gametophytes (a) and of 6-wk-old cultures of gametophytes exposed continuously for 4 wk to 100 ppb copper (b) and 200 ppb copper (c).

Effects of Copper on Egg Production

The effects of low-range copper concentrations on the growth of various stages of the vegetatively growing gametophytes was not tested. The effects on gametophyte "viability," i.e., the ability of the gametophyte to produce eggs, was tested only for continous exposure to low-range copper concentrations.

In Experiments M, N, and O, continuously exposed gametophytes were followed through sexual maturation (see Table 20 for results). Eggs rather than antherozoids were counted because the antherozoids are small (less than 5 μm in diameter) and motile, whereas the eggs are large (20 μm in diameter) and nonmotile. Copper spikes of 30 and 50 ppb inhibited the production of eggs, but spikes of 10 and 20 ppb copper stimulated the production of eggs, relative to controls. A graph of the total number of eggs that crossed the micrometer line vs concentration is presented in Fig. 14.

Copper concentrations in the experimental medium were determined by DPASV (see Table 21). The large standard deviations for the total and labile measurements of the 10-ppb copper spike were consistent with those found for the vegetatively growing cultures. The total copper measured for the 20-ppb spike was lower than that for the vegetatively growing cultures (5 vs 19 ppb copper), although the less-than-1-ppb value for the labile portion was consistent with

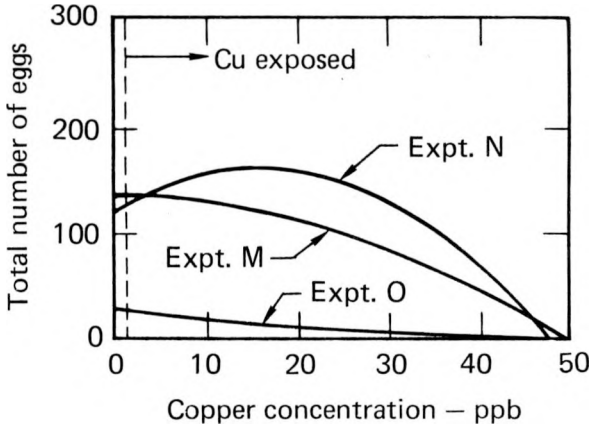


Fig. 14. Graph showing the egg productions by female gametophytes vs copper concentration for gametophytes exposed continuously to low-range copper concentrations.

Table 20. Composite of results for low-range copper concentrations, Experiments M, N, and O.

Copper concentration, ppb	Mean number of eggs crossing micrometer line ± std. dev. (percent inhibition)		
	Experiment M	Experiment N	Experiment O
0 (control)	33.3 ± 35.6 (0)	24.3 ± 11.9 (0)	7.0 ± 3.5 (0)
10	45.3 ± 18.5[a] (0)	71.0 ± 110.2[b] (0)	10.7 ± 7.5[a] (0)
20	45.0 ± 30.0[a] (0)	85.3 ± 37.5[b] (0)	2.7 ± 4.6[c] (61)
30	19.7 ± 15.0[b] (41)	0.0[c] (100)	0.0[c] (100)
50	0.0[b] (100)	0.0[c] (100)	0.0[b] (100)

[a] Number of eggs does not differ significantly from controls.
[b] Number of eggs differs significantly from controls (Dunnett's test, $p = 0.05$).
[c] Number of eggs differs significantly from controls (Dunnett's test, $0.05 > p > 0.15$).

Table 21. Low-range copper concentrations, Experiments M, N, and O chemical analyses.

Copper concentration, ppb	Sum of number of eggs crossing micrometer line (measured copper, ppb DPASV total/labile)		
	Experiment M	Experiment N	Experiment O
0 (control)	130	73	21
	(–)[a]	(3.9/0.8)	(5.1/1.1)
10	136[b]	213[c]	32[b]
	(–)	(6.8 ± 0.9/3.6 ± 1.5)	(7.7 ± 5.4/3.4 ± 3.0)
20	135[b]	256[c]	8[b]
	(–)	(–)	(5.1/0.4)
30	59[c]	0[c]	0[c]
	(–)	(–)	(18.8/1.2)
50	0[c]	0[c]	0[c]
	(–)	(–)	(14.7/2.4)

[a] Water sample not analyzed.
[b] Number of eggs does not differ significantly from controls.
[c] Number of eggs differs significantly from controls (Dunnett's test, p = 0.05).

that measured in the vegetatively growing cultures. The DPASV measurement of total copper for the 30-ppb copper cultures was somewhat higher than, although consistent with, that of the vegetatively growing cultures (9 vs 13 ppb copper). Like the concentrations determined in the vegetatively grown cultures, the DPASV-labile copper in algae-exposed medium of Experiment O was in the 1-ppb range. Measurements by DPASV of the total (15 ppb) and labile (2 ppb) copper in media spiked with 50 ppb were consistent with those found in the experiments of copper-exposed, vegetatively growing gametophytes.

1-wk Pulsed Exposure

The third type of experiment involved pulsed exposure of gametophytes to mid-range copper concentrations. High-range copper concentrations were not tested because continuous-exposure experiments at these concentrations resulted in deterioration of the plant material within 1 wk. Low-range copper concentrations were not tested because results of the continuous-exposure experiments suggested that no detectable effect would be found.

Spores were exposed for 1 wk to mid-range copper concentrations. Table 22 presents the biological data from Experiment P. The mean number of branches crossing the line for all cultures spiked with 50 to 200 ppb copper was significantly different than the controls for 1 or 2 wk after the spores were placed in control medium. Although the growth-inhibition pattern was not as evident as that seen for plants which were continuously exposed, a graph of the results of this experiment (Fig. 15) reveals that 3 wk of growth in the control medium was necessary before the growth of spiked cultures was the same as the controls.

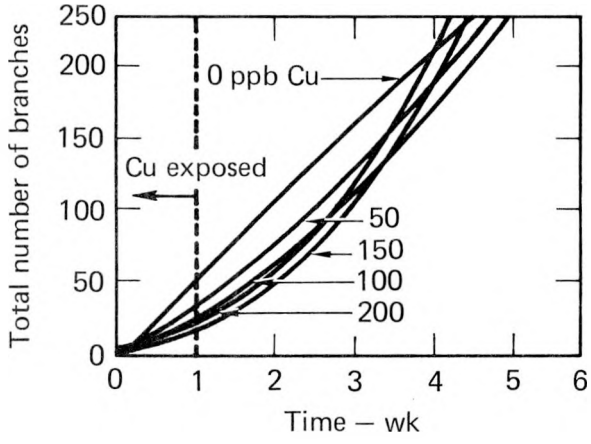


Fig. 15. Graph showing the growth of spores after a pulsed exposure to mid-range copper concentrations vs time.

Table 22. Results of mid-range copper concentrations, Experiment P: pulsed exposure of spores.

Copper concentration, ppb[a]	Mean number of branches crossing micrometer line ± std. dev. (percent inhibition)				
	Week 1	Week 2	Week 3	Week 4	Week 5
0 (control)	15.7 ± 1.2 (0)	34.7 ± 9.0 (0)	63.0 ± 6.9 (0)	67.7 ± 5.7 (0)	50.3 ± 4.6 (0)
50	13.3 ± 2.1[b] (15)	26.7 ± 5.7[b] (23)	43.0 ± 12.2[c] (32)	64.7 ± 4.2[b] (4)	49.0 ± 4.4[b] (3)
100	9.0 ± 1.7[c] (43)	21.0 ± 6.1[b] (39)	39.7 ± 3.8[c] (37)	57.0 ± 6.2[b] (16)	48.0 ± 4.0[b] (5)
150	9.3 ± 2.5[c] (41)	15.0 ± 2.6[c] (57)	34.7 ± 4.6[c] (45)	63.7 ± 6.0[b] (6)	46.3 ± 12.5[b] (8)
200	14.7 ± 2.1[b] (6)	13.0 ± 5.2[c] (63)	40.3 ± 5.9[c] (36)	69.7 ± 6.5[b] (0)	52.0 ± 2.0[b] (0)

[a] Water samples not analyzed for this experiment.
[b] Growth not statistically different than controls.
[c] Growth differs significantly from controls (Dunnett's test, p = 0.05).

DISCUSSION

Copper toxicity to the gametophytes of *Macrocystis pyrifera* can occur on several physiological levels, and death of the plant may result from a combination of factors. By hypothetically tracing the movement of a copper ion from the surrounding medium into a plant cell, we discuss the various systems that could be affected by the presence of a chemically active heavy metal.

The cell wall of the *Macrocystis* gametophyte is a complex structural polysaccharide covered by a layer of mucus. These polysaccharides are known to bind heavy metals. Studies using gels of sodium alginate (a water soluble extract of brown algae) have shown that upon exposure to various heavy metals, stable metal salts are formed that congeal the gel in direct proportion to the bound ion content (Seeley and Hart, 1974). Although Seeley and Hart did not include a specific analysis of the effect of copper exposure on the alginate gel, one would expect copper to have the same effect.

In the gelatinous mucus layer surrounding the cell wall, copper also could react to congeal the mucus into a more dense polysaccharide. Such a change in the protective mucus lining could affect the plant by restricting the ability of trace metals to move through the mucus to the cell wall. Over time, this restriction would produce a nutrient deficiency. Also by changing the structure of the polysaccharide via cross linking between polymer units, the solubility of the mucus layer could be reduced. This solubility change could have an adverse effect on the ability of the cell to chelate metal ions in the medium by exuding fixed carbon.

Seeley and Hart (1973) further demonstrated that alginate preferentially binds bivalent ions in the following order: lead and cadmium over barium over calcium and strontium over magnesium. In addition, preferential binding of copper over cadmium and zinc has been demonstrated for *Ulothrix*. Although the cell walls of this green alga are composed of cellulose, not alginic acids, the stability of the copper-ligand complexes is believed to be greater than that of most other heavy metal-ligand complexes except lead or mercury (Ferguson and Bubela, 1974). If copper ions displace the metal ions bound in the cell wall, trace metal

deficiences theoretically could develop where copper had replaced some essential mineral.

Because the cell wall is integrally associated with the cell membrane, increased accumulation of copper in the cell wall could upset the delicate ionic balance of the cell membrane. According to current theory, copper ions outside the cell cause the loss of potassium ions through the cell membrane, thus disrupting the sodium pump (Overnell, 1975). Once membrane integrity has been lost, incursion of the copper into the cell can occur.

Copper can bind increasingly to the cell membrane, disrupting the membrane structure and causing the loss of cell contents. This was observed when we exposed *Macrocystis* gametophytes to copper concentrations of 500 ppb or more. At lower copper concentrations, traumatic expulsion of the cell contents does not occur, but copper can deactivate key physiological systems (Nielsen and Wium-Anderson, 1970; Nielsen and Kamp-Nielsen, 1970).

The photosynthetic system of algae is vulnerable to heavy metal interference because copper is a constituent of plastocyanin and cytochrome oxidase, key components of the electron transport chain. Indirect interference by copper with the elements of the photosynthetic apparatus could occur at the sodium pump of a chloroplast membrane; copper is expected to behave here in a manner similar to that hypothesized for its interference with the cell membrane pump (Overnell, 1975). Direct interference by copper competing for metal binding sites in the cytochromes (iron) or in the chlorophyll molecules (magnesium) also might lead to a disruption of photosynthesis (Hill, 1963). Chlorophyll changes and carotenoid destruction are known to occur when extracts of the pigments of *Chlorella* are exposed to 10^{-3} *M* (1000 ppb) copper for as little as 10 min (Gross, *et al.*, 1970). In addition, pure extracts of B-carotene, the major pigment of both brown and green algae, break down in less than 30 min when exposed to 10^{-4} *M* (100 ppb) copper (Gross, *et al.*, 1970).

Copper interference with key enzyme systems can occur both directly and indirectly. Competitive or noncompetitive inhibition of metal cofactor binding sites on enzyme or substrate molecules is an example of direct interference with a given enzyme system. Mineral deficiences caused by the preferential binding of copper to cell walls constitute an indirect type of interference with enzyme function.

Acute Toxicity Response of *Macrocystis* Gametophytes

At copper concentrations of 500 ppb or more, disintegration of plant material was observed for continuously exposed cultures. The primary toxic response was rupture of the cell membrane and extrusion of the cell contents. At copper concentrations above 500 ppb, the analyses for total copper in the stored water samples did not show a consistent pattern of copper removal, as was expected from the interaction between the cationic binding sites on the culture vessel walls and the copper in the medium. At the higher concentrations, some copper may have been lost by precipitation and, consequently, may not have been transferred to the stored water sample. The poor recoveries at the higher concentrations also may have been the result of an interaction between the stored water sample (at pH 2) and the walls of the polyethylene storage bottle. Polyethylene can react with acid over a period of time and dissolve to some extent, creating potential binding sites for metal ions.

Exposure of the gametophytes to mid-range copper concentrations inhibited growth in varying degrees of severity. The direct proportionality of growth inhibition to increases in copper concentration as well as the existence of a biomass-related response at a given concentration level imply that, at least in the 100-to-200-ppb range, there is no functional regulation of copper influx.

The severity of copper interference with plant metabolic processes is exhibited by the responses of the variously aged gametophytes that were exposed continuously to copper concentrations between 100 and 200 ppb. In all cases, the continuous exposure of 1-, 2-, and 5-wk-old gametophytes to 200 ppb copper resulted in essentially no growth after the time of initial exposure. In most cases, the number of filaments counted declined toward the end of the experiments; because gametes were not produced, this decline cannot be attributed to the usual disintegration that follows the production of sex products. Therefore, we conclude that the primary toxic effects of 200 ppb copper on *Macrocystis* gametophytes are (1) severe interference with the photosynthetic and respiratory processes and (2), in cases where filament number was reduced, loss of cell membrane integrity (also characteristic of the higher copper concentrations).

Exposure of variously aged, vegetatively growing gametophytes to 100 ppb copper inhibited growth somewhat less severely than the higher copper concentrations. During exposure to 100 ppb copper, photosynthetic gains were measured (as an increase in filament number), suggesting that fixed carbon was being exuded by the gametophytes to complex some of the copper in the medium.

The chemical data for the mid-range copper experiments show that the percent recoveries for copper concentrations between 100 and 200 ppb were consistently near 75%. The apparent "loss" of copper could have resulted from (1) adsorption of copper onto the walls of the culture vessels, (2) adsorption of copper onto the walls of the storage containers, (3) adsorption and absorption of the copper by the algae, and (4) precipitation of copper out of the medium in metal-organic complexes.

A biomass-related pattern appeared when the results of the three experiments exposing vegetative gametophytes of varying ages to mid-range copper concentrations were compared. Younger gametophytes (1 to 2 wk old) have a smaller biomass than the older gametophytes (5 wk old). Consequently, the number of possible binding sites and the ability of the gametophyte to exude fixed carbon probably are less in the younger plants. As a result, the ability of the gametophyte to detoxify the medium by binding or complexing the biologically active copper would be less in the 1- and 2-wk-old plants than in the 5-wk-old gametophytes. This suggestion is supported by data which show that 1- and 2-wk-old gametophytes are inhibited 50 and 38% respectively, whereas 5-wk-old plants are inhibited 29% by a 1-wk exposure to 200 ppb copper.

Continuous exposure to copper concentrations between 50 and 100 ppb not only resulted in an inhibition of vegetative growth, but also in a qualitative change in the appearance of exposed cultures. Microscopic examination of the spiked cultures revealed that the sexual characteristics of gametophytes continuously exposed to nominal concentrations of 50 and 75 ppb copper were less clearly defined than those of the controls. Whether this "masking" of sexual characteristics was real or a manifestation of selective pressure against one of the sexual types is a question that could be resolved by exposing unisexual cultures to various copper concentrations.

Another anomaly observed in the copper-exposed gametophytes was the tendency to form ramified filaments, rather than the uniaxial filaments typical of control cultures. Although the role of growth hormones in *Macrocystis pyrifera* has not been firmly established, auxin activity in this alga has been reported (Provasoli and Carlucci, 1974). If branching of the filaments is hormonally governed, as in higher plants, specific interference of copper with a growth-regulating hormone system is possible.

Growth Strategy of Mid-Range Exposures

If all obviously contaminated water samples are excluded from our calculations, the percent recoveries for the 50- and 75-ppb copper spikes are 48 and 57%, respectively. In contrast, for the other mid-range concentrations (25, 100, 150, and 200 ppb), the percent recovery is approximately 75%. If this drop in percent recovery is a function of increased adsorption or absorption of copper by the gametophytes, a "growing strategy" can be proposed for the mid-range spikes.

At copper concentrations below a critical level, 50-ppb initial spike, the gametophytes appear to function with little metabolic impairment caused by the copper. The natural "complexing capacity" of the seawater, including such metal-binding moieties as protein, carbohydrate, and lipid molecules, can complex most of the biologically active copper. The remaining active copper probably is bound in the cell walls of the gametophytes and is complexed with algal exudates. The gametophytes thus effectively remove the toxic forms of the metal from the medium. Both the biomass of the gametophytes and the percent recoveries of the copper spikes are expected to be relatively high because the metal would be complexed by the extracellular material in the medium.

At concentrations above this critical level, 50- and 75-ppb copper initial spikes, the complexing capacity of the water is exceeded. Thus the biologically active copper is bound increasingly by the plant material and interferes with the metabolism of the gametophytes. Because the life functions of the plant are not completely halted, some photosynthetic gains can be measured in terms of filament

production, and an increased number of potential binding sites are therefore created as cell wall material is laid down. The biomass of the gametophytes is expected to be somewhat reduced from control levels because of some growth inhibition and the percent recoveries are expected to be lower because of increased copper absorption and adsorption by the gametophytes.

At increasingly higher copper concentrations, 100-, 150-, and 200-ppb initial spikes, the ability of the plants to detoxify the medium by exuding fixed carbon is reduced. Severe interference with photosynthesis and respiration further reduces the production of cell wall material and thus decreases the number of potential binding sites for the uncomplexed metal. Because the biomass of the gametophytes is expected to be small, there should be fewer potential binding sites. The percent recoveries of the copper spikes are expected to be high because relatively little copper will be bound to plant material.

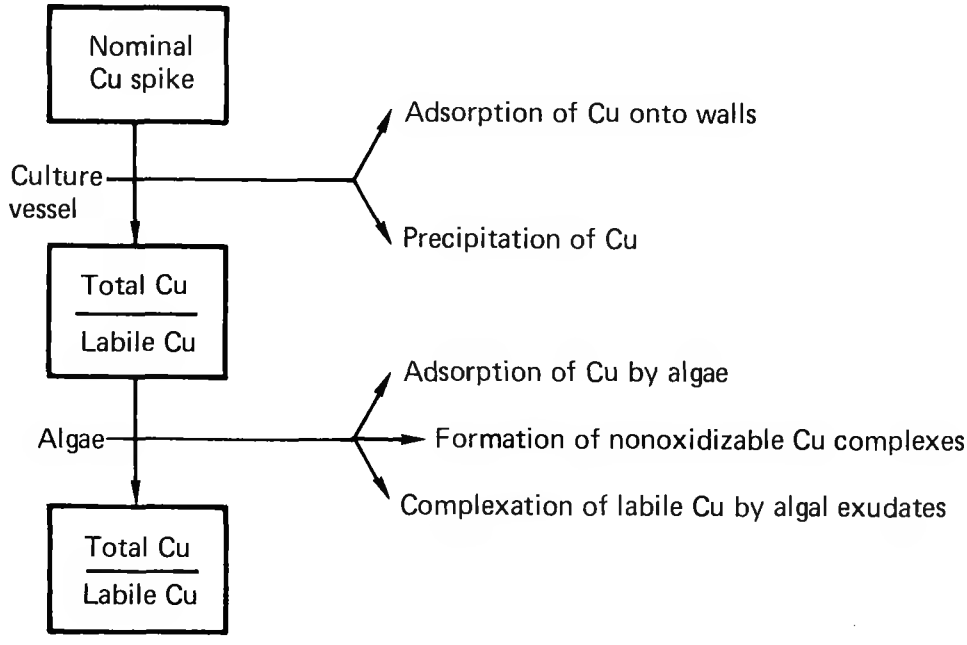
Growth Strategy and Copper Speciation

Growth strategies, not metabolic regulation, may account for the observed changes in copper recoveries at nominal concentrations of less than 50 ppb copper. The DPASV analyses suggest a complex interaction among the algae, medium, and culture vessel. The mean percent recoveries for the low-range experiments were generally higher than those for the mid-range experiments. This may be partially due to the fact that the DPASV analyses were performed on fresh samples that had not been stored. The consistency of the standard deviation for the percent recovery (except for the 50-ppb copper samples) suggests a relatively fixed number of binding sites on the culture vessel surfaces. However, it is possible that this deviation may have been somewhat a function of pipetting errors, because very small volumes of stock solution (10 to 50 μlitre) were added to make the media.

The changes in the characteristics of the medium after a 1-wk exposure to the gametophytes reveal the dynamics of the interaction between the plants and copper. Of the approximate 11-ppb total copper in the 10-ppb spiked medium, about 7 ppb were recovered after exposure to the plants (see Fig. 16). The 4 ppb copper not recovered probably was adsorbed by the plants and complexed into nonoxidizable copper chelates. Measurements of total copper in these water samples by graphite furnace atomic absorption spectrophotometry gave a mean recovered copper concentration of 7.8 ppb, suggesting that tightly bound copper complexes are not an important loss mechanism at this low copper level (10 ppb). Of the approximate 4.5-ppb ASV-labile copper available to the plant, about 3 ppb remained labile after the 1-wk exposure to the gametophytes. However, the standard deviations for both means were high enough to suggest that little or no ASV-labile copper remained in the medium after storage in the culture vessels, whether or not gametophytes were present.

For nominal spikes of 20 ppb copper, approximately 9 ppb was recovered with the DPASV analysis method of perchlorate-digested, organic-copper complexes in gametophyte-exposed medium. Measurements of total copper by graphite furnace atomic absorption spectrophotometry gave a mean recovered copper content of 11.3 ppb, suggesting again that "loss" of copper to nonoxidizable organic-copper complexes is not a major factor. Although the mean value for labile copper was approximately 11 ppb, the standard deviation was sufficiently high to suggest that little or no ASV-labile copper was available after a 1-wk exposure to the glass culture vessels. After exposure to the gametophytes, little or no ASV-labile copper remained in the culture medium with nominal spikes of 20 ppb copper.

For culture medium spiked with 30 ppb copper, of which approximately 26 ppb was recovered after perchlorate digestion, only about 13 ppb was recoverable from cultures containing algae. Analyses of total copper by graphite furnace atomic absorption spectrophotometry gave an average of 10 ppb copper for these samples. Again, this suggests that "loss" of copper to nonoxidizable complexes is not significant. The mean labile copper concentration available to gametophytes was 20 ± 11 ppb (see Fig. 16). This suggests that the binding sites of the culture vessel and the natural complexing capacity of the seawater from which the medium was made were saturated, and that the medium contained an excess of ASV-labile copper. Growth of algae for 1 wk in the medium resulted in the complexing of a considerable portion of labile copper; the content of ASV-labile copper dropped to about 1 ppb.



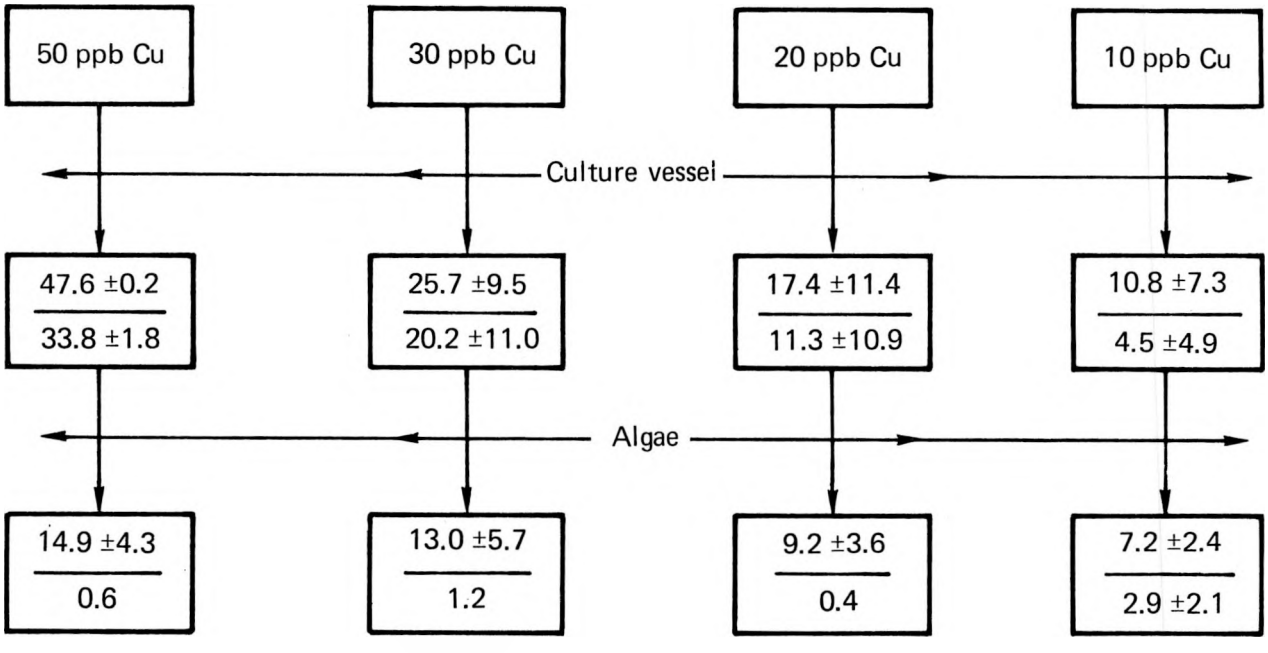


Fig. 16. Diagram of the partitioning of labile copper in culture medium of low-range copper concentrations.

For the 50-ppb copper spikes, about 48 ppb was detected after storage for 1 wk in the culture vessels; nearly 34 ppb of this copper remained ASV-labile. Total copper in the medium, measured by DPASV, was approximately 15 ppb after a 1-wk exposure to the gametophytes. Analysis of this water sample by graphite furnace atomic absorption spectrophotometry gave a total recovered copper content of about 16 ppb, again suggesting that the forms of the copper complexes were perchlorate-digestable. After 1 wk of growth in the copper-spiked medium, the algae had complexed essentially all of the ASV-labile copper (see Fig. 16).

The proposed mechanisms for the interaction of the gametophytes with the biologically active copper in the medium are further supported by examining the percent recoveries for the low-range, copper-exposure experiments. The percent recoveries declined as the concentration of the initial spike increased from 10 to 50 ppb copper. This decline can be attributed to increased adsorption and absorption of copper by the gametophytes.

Sexual Maturation and Copper Speciation

The effect of ASV-labile copper on the production of eggs by the gametophytes followed the pattern set by the plants in their vegetative state: where a significant amount of labile copper remained, egg production and vegetative growth were inhibited; where copper was complexed, egg production and vegetative growth were enhanced. However, the effects of copper on the viability of exposed eggs, i.e., the production of the sporophyte generation, needs further study.

The stimulation of egg production and vegetative growth relative to controls at the lowest copper concentrations suggests a growth requirement for copper in a chelated form and a synergistic interaction with or a substitution for a metallic nutrient that was not included in our control medium. As discussed earlier, iron was eliminated from the trace metal enrichment, and molybdenum, included by some researchers, (Sanbonsugu, personal communication, 1978; West, personal communication, 1978), was not included.

Pulsed-Exposure Recovery

The recovery of the gametophytes from a 1-wk exposure to mid-range copper concentrations generally required about 3 wk. However, the pattern of biomass-related differences in the recovery period was not as well defined as expected. By comparing the number of filaments counted for two consecutive weeks at a given copper concentration, a growth response pattern appears. At the highest concentrations, 150 and 200 ppb copper, the number of filaments produced during the first week after removal of the metal-induced stress was unchanged for the gametophytes exposed to 200 ppb copper and increased by a factor of 1.6 for plants exposed to 150 ppb copper. The filament increase factor for the control gametophytes was 2.2. After 1 wk, the increase values for gametophytes exposed to 50 and 100 ppb copper were 2.0 and 2.3, respectively. This suggests that the type or level of toxic response is, as expected, more severe at higher copper concentrations.

However, an unexpected result appeared in this experiment. Some of the metal-stressed gametophytes grew more rapidly than the controls in the second and third weeks after they were transferred to the control medium. Perhaps there was a mineral deficiency in the control medium for which copper-exposed gametophytes were able to compensate. Whether these metal-stressed gametophytes would have been able to mature sexually is a question that has yet to be resolved.

CONCLUSION

The physiological toxic effects of copper on *Macrocystis* gametophytes can be divided into three general categories. First, acute copper toxicity is characterized by traumatic expulsion of the cell contents. This is probably the result of cell membrane disintegration and was observed at spikes of 500 ppb copper or more.

Second, acute copper toxicity is characterized by varying degrees of growth inhibition, resulting from the interference of copper with photosynthesis, respiration, and enzyme function. At copper concentrations nominally between 50 and 200 ppb, when the metal-induced stress is continued for 4 to 6 wk, the gametophytes disintegrate at a rate directly related to the amount of copper in the medium. For pulsed exposures of 1-wk-old gametophytes to mid-range copper concentrations, the recovery of vegetative growth to control levels takes approximately 2 to 3 wk. Whether sexual maturation is impaired by the metal-induced stress during the vegetative phase is a question that needs to be answered.

Third, subacute toxic effects were observed with initial spikes of 30 and 50 ppb copper. At these levels, initial concentrations of ASV-labile copper (probably less than 10 ppb) block the production of eggs in female gametophytes. Whether this interference occurs at one or several key enzymatic reactions is unknown. With initial copper spikes of 10 and 20 ppb, little or no ASV-labile copper was available to the gametophytes because the biologically active metal forms are readily complexed by the ligands naturally present in the seawater and by those ligands exuded by the plant.

Further experiments in a flow-through bioassay system, rather than our dish-culture system, should solve the problem of maintaining a more constant level of ASV-labile copper. Although the acute toxicity levels for *Macrocystis* gametophytes have been successfully established with the dish-culture bioassay, the subacute effects of copper on *Macrocystis* gametophyte sexual maturation need to be tested in a flow-through system.

ACKNOWLEDGMENTS

The authors thank M. Foster, J. Koskin, R. Jim, J. Dawson, Y. Sanbonsugu, and E. Pullen for invaluable assistance.

REFERENCES

Bellamy, D. (1972), "Eyes Beneath the Waves," *New Sci. 13* Apr, 76–78.

Berland, B. R., D. Bonin, V. Kapkov, S. Maestrini, and D. Arlhae (1976), "Action Toxique de Quatre Lourds sui la Croissance d'Algues Unicellulaires Marines," *C. R. Acad. Sci.* t 282, Series D, 633–636.

Boyle, E., and J. M. Edmond (1975), "Copper in Surface Waters South of New Zealand," *Nature 253*, 107–109.

Bryan, G. W. (1969), "The Absorption of Zinc and Other Metals by the Brown Seaweed *Laminaria digitata*," *J. Mar. Biol. Assoc. U. K. 49*, 225–243.

Bryan, G. W., and L. G. Hummerstone (1973), "Brown Seaweed as an Indicator of Heavy Metals in Estuaries in South-West England," *J. Mar. Biol. Assoc. U. K. 53*, 705–720.

Byrne, R., and D. R. Kester (1976), "Solubility of Hydrous Ferric Oxide and Iron Speciation in Seawater," *Mar. Chem. 4*, 255–274.

Chester, R., and J. H. Stoner (1974), "The Distribution of Zinc, Nickel, Manganese, Cadmium, Copper and Iron in Some Surface Waters from the World Ocean," *Mar. Chem. 2*, 17–32.

Clendenning, K. A., and W. J. North (1960), "Effects of Wastes on the Giant Kelp, *Macrocystis pyrifera*," in *Proceedings of First International Conference on Waste Disposal in the Marine Environment*, E. A. Pearson, Ed. (Pergamon Press, New York), pp. 82–91.

Dorband, W. R., J. Van Olst, J. Carlberg, and R. Ford (1975), "Effects of Chemicals in Thermal Effluent on *Homarus americanus* Maintained in Aquaculture Systems," Contribution No. 15 from the San Diego State University Center for Marine Studies.

Dunstan, W. M. (1975), "Problems of Measuring and Predicting Influence of Effluents on Marine Phytoplankton," *Envir. Sci. Technol. 9*(7), 635–638.

Erickson, S. J., *et al.* (1970), "A Screening Technique for Estimating Copper Toxicity to Estuarine Phytoplankton," *Jour. Wat. Poll. Cont. Fed. 42*(8–2), R270–R278.

Ferguson, J., and B. Bubela (1974), "The Concentration of Cu(II), and Pb(II) and Zn(II) from Aqueous Solutions by Particulate Algal Matter," *Chem. Geol. 13*, 163–186.

Gherardini, G. L., and W. J. North (1972), "Electronmicroscopic Studies of *Macrocystis pyrifera* Zoospores, Gametophytes, and Early Sporophytes," in *Proc. 7th Intl. Seaweed Symp.*, Sapporo, Japan (1971), pp. 1972–1980.

Gross, R. E., P. Pugno, and W. M. Cugger (1970), "Observations on the Mechanism of Copper Damage in *Chlorella*," *Plant Physiol. 46*(2), 183–185.

Hassal, K. A. (1967), "Inhibition of Respiration of *Chlorella vulgaris* by Simultaneous Addition of Cupric and Fluoride Ions," *Nature, Lond. 215*, 521.

Helz, G. R., R. J. Huggett, and J. M. Hill (1975), "Behavior of the Mn, Fe, Cu, Zn, Cd, and Pb Discharged from a Wastewater Treatment Plant into an Estuarine Environment," *Water Res. 9*, 631–636.

Hill, R. (1963), "Chlorophyll," in *Comprehensive Biochemistry*, M. Florkin and E. H. Stotz, Eds. (Elsevier Publishing Co., Amsterdam), Vol. 9, pg. 73.

Hutchinson, G. E. (1957), "Minor Metallic Elements in Lake Waters," in *Treatise on Limnology*, (John Wiley & Sons, Inc., New York), Vol. 1, pp. 811–820.

Jensen, A., and B. Rystad (1976), "Heavy Metal Tolerance of Marine Phytoplankton II. Copper Tolerance of Three Species in Dialysis and Batch Cultures," *J. Exp. Mar. Biol. Ecol. 22*, 249–256.

Khailov, K. M. (1964), "The Formation of Organometallic Complexes with the Participation of External Metabolities of Marine Algae," *Hydrobiol. 154*(1–6), 237–239.

Khobot'ev, V. G. (1975), "Toxicity of Copper Containing Compounds for Algae," *Girdrobiol. Zh. 11*(5), 49–55 (abstract).

Knauer, G. A., and J. H. Martin (1973), "Seasonal Variations of Cadmium, Copper, Manganese, Lead, and Zinc in Water and Phytoplankton in Monterey Bay, California," *Limnol. Oceanog. 18*(4), 597–604.

Manahan, S. E., and M. J. Smith (1973), "Copper Micronutrient Requirement for Algae," *Envir. Sci. Technol. 7*(9), 829–833.

Martin, M. (1974), *A Summary of the Cause and Impact of an Abalone Mortality–Diablo Cove, San Luis Obispo County, California*, Marine Resources Region, California Department of Fish and Game.

Martin, M., M. D. Stephenson, and J. H. Martin (1974), "Copper Toxicity Experiments in Relation to Abalone Deaths Observed in a Power Plant's Cooling Water," Manuscript in preparation.

Morris, A. W. (1974), "Seasonal Variation of Dissolved Metals in Inshore Waters of the Menai Straits," *Mar. Poll. Bull. 5*(4), 54–59.

Morris, A. W., and A. J. Bale (1975), "The Accumulation of Cadmium, Copper, Manganese and Zinc by *Fucus vesiculosus* in the Bristol Channel," *Est. Coast. Mar. Sci. 3*, 153–163.

Morris, O. P., and G. Russel (1973), "Effect of Chelation on Toxicity of Copper," *Mar. Poll. Bull. 4*(10), 159–160

Nielsen, L. K. (1969), "The Influence of Copper on the Photosynthesis and Growth of *Chlorella pyrenoidosa*," *Physiol. Plant. 22*, 249–254.

Nielsen, S., and S. Wium-Anderson (1970), "Copper Ions as Poisons in the Sea and in Fresh Water," *Mar. Biol. 6*, 93–97.

Nielsen, S., and L. Kamp-Nielsen (1970), "Influence of Deleterious Concentrations of Copper on the Growth of *Chlorella pyrenoidosa*," *Physiol. Plant. 23*, 828–840.

Nielsen, S., and S. Wium-Anderson (1971), "The Influence of Cu on Photosynthesis and Growth in Diatoms," *Physiol. Plant. 24*, 480–484.

North, W. J. (1977), "Growth Factors in the Production of Giant Kelp," presented at *IGT Symposium on Clean Fuels from Biomass and Wastes*, Orlando, Florida.

O'Dom, G. W., and H. D. Siegerman, Eds. (1973), *Modern Analytical Polarography Workshop Manual*, Princeton Applied Research Corporation.

Overnell, J. (1975). "The Effect of Heavy Metals on Photosynthesis and Loss of Cell Potassium in Two Species of Marine Algae, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*," *Mar. Biol. 29*, 99–103.

Paraschiv, M. (1975), "Effect of Relatively High Concentrations of Trace Element on the Biomass, Morphology and Growth Rhythm Intensity in *Scenedesmus quadricauda* and *Chlorella vulgaris* Algae," *Stud. Cercet. Biol. 27*(3), 219–225 (abstract).

Pigman, W., D. Horton, and A. Herp, Eds. (1970), *Carbohydrates–Chemistry and Biochemistry* (Academic Press, New York), Vol. II B.

Prakash, A., and M. A. Rashid (1968), "Influence of Humic Substances on the Growth of Marine Phytoplankton: Dinoflagellates," *Limnol. Oceanog. 13*, 598–606.

Preston, A., D. Jeffries, J. Dutton, B. Harvey, and A. Steele (1972), "British Isles Coastal Waters: The Concentrations of Selected Heavy Metals in Sea Water, Suspended Matter and Biological Indicators–A Pilot Survey," *Envir. Poll. 3*, 69–82.

Provasoli, L., and A. F. Carlucci, "Vitamins and Growth Regulators," in *Algal Physiology and Biochemistry*, W. D. P. Stewart, Ed. (University of California Press, Berkeley), 989 pp.

Quagliano, J. V. (1964) *Chemistry* (Prentice-Hall, Inc., Englewood Cliffs, NJ), p. 432.

Rosenthal, R. J., W. D. Clarke, and P. K. Dayton (1974), "Ecology and Natural History of a Stand of Giant Kelp, *Macrocystis pyrifera*, off Del Mar, California," *Fish. Bull., U. S. 72*, 670–684.

Seeley, G. R., and R. L. Hart (1974), "The Binding of Alkaline Earth Metal Ions to Alginate," *Macromol. 7*(5), 706–710.

Seeliger, U., and P. Edwards (1977), "Correlation Coefficients and Concentration Factors of Copper and Lead in Seawater and Benthic Algae," *Mar. Poll. Bull. 8*(1), 16–19.

Sick, L. V., and H. L. Windom (1975), "Effects of Environmental Levels of Mercury and Cadmium on Rates of Metal Uptake and Growth Physiology of Selected Genera of Marine Phytoplankton in Mineral Cycling in Southeastern Ecosystems," *ERDA Symposium Series*, Oak Ridge, TN, USERDA, Technical Information Center, pp. 239–249.

Slowey, J. F., and D. W. Hood (1971), "Copper, Manganese and Zinc Concentrations in Gulf of Mexico Waters," *Geochim. Cosmochim. Acta 35*, 121–138.

Spencer, D. W., and P. G. Brewer (1969), "The Distribution of Copper, Zinc and Nickel in Sea Water of the Gulf of Maine and the Sargasso Sea," *Geochim. Cosmochim. Acta 33*, 325–339.

Sunda, W., and R. R. L. Guillard (1976), "The Relationship between Cupric Ion Activity and the Toxicity of Copper to Phytoplankton," *Jour. Mar. Res. 34*(4), 511–529.

Watson, D. J. (1976), *Copper in the Marine Environment and its Effect on the Green Alga, Ulva lobata*, Masters Thesis, California State University, Hayward, 83 pp.

Wort, D. J. (1955), "The Seasonal Variation in Chemical Composition of *Macrocystis integrifolia* and *Nereocystis luetkeana* in British Columbia Coastal Waters," *Can. J. Botany 33*(4), 323–340.

Yamamoto, T., and M. Ishibashi (1972), "The Content of Trace Elements in Seaweeds," in *Proc. 7th Intl. Seaweed Symp.*, Sapporro, Japan (1971), pp. 511–514.

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